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Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: UTILIZATION OF PIT EMULSIONS IN FERMENTATION PROCESSES

(54) Bezeichnung: VERWENDUNG VON PIT-EMULSIONEN IN FERMENTATIONSVERFAHREN

(57) Abstract: The invention relates to the utilization of O/W emulsions in fermentation processes, said emulsions containing at least water, emulsifying agents and an oil phase that contains one or more compounds selected from the groups consisting of: a) fatty acid alkyl ester and/or b) triglycerides of vegetable origin, wherein the emulsions are produced according to the PIT method and the emulsions have an average drop size ranging from 50 to 400 nm.

(57) Zusammenfassung: Verwendung von O/W-Emulsionen, enthaltend mindestens Wasser, Emulgatoren sowie eine Ölphase, die einen oder mehrere Verbindungen enthält, ausgewählt aus den Gruppen a) der Fettsäurealkylester und/oder b) der Triglyceride pflanzlichen Ursprungs, wobei die Emulsionen nach dem PIT-Verfahren hergestellt werden und eine mittlere Tröpfchengröße im Bereich von 50 bis 400 nm aufweisen, in Fermentationsverfahren.



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## Utilization of PIT Emulsions in Fermentation Processes

This invention relates to the use of emulsions produced by the PET method in fermentation processes.

Microbiological processes are being increasingly used in the synthesis of complex natural substances and other organic compounds. 5 Such processes involve a conversion/transformation under anaerobic or aerobic conditions in which microorganisms, but especially bacteria or fungi, participate. Various terms - not always clearly distinguished from one another (such as bioconversion, biotransformation, fermentation) - are used by experts for microbiological processes. The term "fermentation" is used 10 in the present specification for processes where microorganisms, preferably bacteria, are used for the transformation or synthesis of chemical compounds.

An important element in the development and optimization of fermentation processes is in particular the reaction medium in which the 15 microbiological transformation takes place. The reaction medium, generally an aqueous solution or dispersion, influences above all the yield and efficiency of the process. The microorganisms need carbon, nitrogen and certain trace elements in bound form, for example calcium, iron, phosphorus or zinc, as nutrients to make successful metabolization to the 20 required products possible. In addition, the temperature and pH regularly have to be kept in a certain, generally narrow range. Further details can be found in the manual by W. Crueger/A. Crueger, **Biotechnologie - Lehrbuch der angewandten Mikrobiologie, 2nd Edition 1984, R. Oldenbourg Verlag**. Chapter 5 of this work is particularly concerned with 25 the fundamentals of fermentation. Accordingly, this literature reference also belongs specifically to the disclosure of the present invention. Besides high-energy sugars and derivatives thereof, natural fats and oils and

derivatives thereof, such as glycerol, glycerides, fatty acids or fatty acid esters, are additionally used as nutrients for the microorganisms in many processes. The culture media may not of course contain any ingredients which could adversely influence the metabolization of the microorganisms.

- 5           **DE 37 38 812 A1**, for example, describes a microbial process for the production of  $\alpha,\omega$ -dicarboxylic acids in which bacteria of the strain *Candida tropicalis* transform methyl laurate into the required dicarboxylic acids. This transformation takes place in an aqueous medium at a pH of 6.0 and at a temperature of 30°C. Besides the microorganisms, the medium contains
- 10 glucose as an energy source, ethoxylated sorbitan monooleate as emulsifier, yeast extract, corn steep liquor and inorganic N and P sources. The methyl laurate is then added to the medium. There is nothing in the document in question to indicate the type of emulsion which forms in the fermenter or in which the methyl laurate is added to the fermentation broth.
- 15           **EP 0 535 939 A1** describes a process for the production of  $\omega$ -9-polyunsaturated fatty acids in which suitable microorganisms produce the required polyunsaturated fatty acids in an aqueous culture medium in the presence of sugars as energy sources and inorganic or organic nitrogen sources and in the presence of fatty acid methyl esters.

- 20           However, other known processes use only fatty compounds of the type described above as energy sources. This is particularly of economic interest because fatty compounds such as these are generally less expensive than sugars, starch and similar compounds. Park et al. (Park et al., **Journal of Fermentation and Bioengineering**, Vol. 82, No. 2, 183-
- 25 **186, 1996**) describe a fermentation process for the production of tylosine in which microorganisms of the strain *Streptomyces fradiae* are used in an aqueous medium, rapeseed oil being present as sole carbon source in starting quantities of about 60 g/l.

- In fermentation processes, the oxygen content in the medium or the
- 30 fermentation broth also plays a key role. In aerobic processes, the oxygen

acts as a substrate. A critical factor is whether a transfer of oxygen from the gas phase to the liquid phase containing the microorganisms can take place sufficiently for the particular process. An important parameter is the specific exchange surface which, in general, is indirectly determined via the oxygen transfer coefficient  $k_{La}$  (cf. *Crueger*, Chapter 5, pages 71 et seq.). Adjustment of the optimum oxygen input is typically achieved by stirring the fermentation broth, the oxygen or the air being mixed with the liquid and the exchange of gas thus taking place at the interfaces. However, the considerable mechanical input of energy by intensive stirring, as carried out by Park et al., can also destroy parts of the culture, thus reducing the yield of the process. In addition, the dead microorganisms are themselves further degraded and can lead to poisoning of the culture through the degradation products formed so that economic production is not possible. It is known from the work of Goma and Rols (G. Goma, J.L. Rols, **Biotech. Let.**, Vol. 13, No. 1, pages 7 to 12, 1991) that the use of soybean oil in fermentation processes for the production of antibiotics leads to an improvement in the oxygen transfer coefficient  $k_{La}$  which, for the same energy input (stirring), can lead to an increase in the yield of the process as a whole.

Now, the problem addressed by the present invention was to improve fermentation processes so that, on the one hand, inexpensive carbon sources could be used and, on the other hand, an adequate supply of oxygen to the microorganisms would be guaranteed without the microorganisms being exposed to unacceptably severe mechanical stressing by stirring. A way was to be found of minimizing the mechanical input of energy in fermentation processes without any reduction in yield. Preferably, the yield would be increased despite the reduced energy input.

It has now been found that the use of special fine-droplet oil-in-water (o/w) emulsions solves the problem stated above.

In a first embodiment, the present invention relates to the use of o/w

emulsions in fermentation processes, the emulsions containing at least water, emulsifiers and an oil phase, the oil phase containing one or more compounds from the groups of

a) fatty acid alkyl esters and/or

5 b) triglycerides of vegetable origin

and the emulsions being produced by the PET method and having a droplet size of 50 to 400 nm.

It is known that oil-in-water (o/w) emulsions produced with nonionic emulsifiers and stabilized can undergo generally reversible phase inversion on heating, i.e. the emulsion can change type from an o/w to a w/o (water-in-oil) emulsion in a certain temperature range. Since the oil becomes the outer continuous phase in the process, the conductivity of the emulsion falls to zero. The mean value of the temperatures between maximum conductivity of the emulsion and zero conductivity thereof on heating is called the phase inversion temperature (PIT) and the emulsions produced in this way are called PIT emulsions. It is also known that the position of the PIT depends on many factors, for example on the type and phase volume of the oil component, on the hydrophilia and structure of the emulsifiers and on the composition of the emulsifier system. The droplet fineness of PIT emulsions is critically determined by the process used for their production. In general, the water and oil phases are mixed with the emulsifiers and then heated to a temperature above the PIT. Conductivity must fall to zero in the process. The emulsion is then cooled to the starting temperature (generally room temperature, ca. 20°C). The emulsion used in accordance with the invention is formed by the temperature first exceeding and then falling below the phase inversion temperature. It is known that only those PIT emulsions which form a microemulsion phase with low interfacial tension between oil and water or a lamellar liquid crystalline phase during the phase inversion process have particularly fine droplets.

30 The critical step in this regard is always the re-inversion on cooling.

**DE 38 19 193 A1** discloses a process for the production of low-viscosity o/w emulsions by the phase inversion technique. In this process, the PIT technique is applied to mixtures containing an oil component, a nonionic emulsifier and a co-emulsifier in aqueous medium. The oil component is said to consist of 50 to 100% by weight of special mono- or diesters, 0 to 50% by weight of C<sub>8-22</sub> fatty acid triglycerides and optionally 0 to 25% by weight of a hydrocarbon oil. Beyond the components mentioned, **DE 38 19 193 A1** does not disclose any other components and does not mention any intended uses of the emulsions produced.

**DE 41 40 562 A1** describes a process for the production of o/w emulsions by the PIT technique in which polar oil components are heated with an emulsifier system containing nonionic emulsifiers with an HLB value of 10 to 18 beyond the PIT temperature of the emulsion in the presence of co-emulsifiers from the group of C<sub>12-22</sub> fatty alcohols and/or Guerbet alcohols and are then cooled again, fine-droplet emulsions being obtained.

**DE 196 35 553 A1** describes emulsifier systems containing fatty acid ethoxylates and partial glycerides as key components for the production of fine-droplet PIT emulsions.

The emulsions according to the invention are distinguished in particular by their droplet fineness. The droplets are between 50 and 400 nm in size, preferably between 100 and 300 nm in size, more preferably between 180 and 300 nm in size and most preferably between 160 and 250 nm in size. The droplet sizes are assumed to have a Gaussian distribution and are measured, for example, by light scattering or absorption.

The fineness of the oil droplets leads to a large surface between the oil and water phases and thus provides for rapid contact between the microorganisms present in the aqueous phase and the oil phase containing the nutrients. The large surface also simplifies the exchange of gases, particularly oxygen and CO<sub>2</sub>. In addition, the viscosity of the emulsion and hence of the entire fermentation medium decreases. As a result, the

stirring speed of the fermentation medium can be distinctly reduced so that the yield of the fermentation process can be increased.

According to the invention, the PIT emulsions are added to the aqueous fermentation medium containing the microorganisms and optionally other auxiliaries and additives. The details of this process, more especially the addition rate and added quantity of the emulsion, are determined by the microorganism strains and the fermentation process selected and may be adapted by the expert to suit the particular circumstances.

Besides water, the PIT emulsions contain an oil phase which contains compounds from the group of fatty acid alkyl esters a) or native vegetable oils and derivatives thereof b). Groups a) and b) are hydrophobic, water-insoluble or substantially water-insoluble compounds which may serve as nutrients, i.e. energy sources, for the bacteria used in the fermentation process, but which may also be starting materials (substrates) for the products to be obtained by bioconversion.

Suitable esters of group a) are derived in particular from saturated, unsaturated, linear or branched fatty acids containing a total of 7 to 23 carbon atoms. In other words, they are compounds corresponding to formula (I):



where  $R^1$  is a  $C_{6-22}$  alkyl group and  $R^2$  is a  $C_{1-4}$  alkyl group. Methyl and ethyl groups are preferred. The use of methyl esters is the most advantageous. The methyl esters of formula (I) may be obtained in known manner, for example by transesterification of triglycerides with methanol and subsequent distillation. Suitable fatty acids are caproic acid, heptanoic acid, caprylic acid, pelargonic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid,

to 9.5, a saponification value of 0.88 to 0.90 and a melting point of 20 to 23°C. Olive oil predominantly contains oleic acid (cf. **Lebensmittelchem. Gerichtl. Chem., 39, 112 to 114, 1985**). Palm oil contains ca. 2% by weight myristic acid, 42% by weight palmitic acid, 5% by weight stearic acid, 5 41% by weight oleic acid, 10% by weight linoleic acid as fatty acid components. Palm kernel oil typically has the following composition in relation to its fatty acid spectrum: 9% by weight caproic/caprylic/capric acid, 50% by weight lauric acid, 15% by weight myristic acid, 7% by weight palmitic acid, 2% by weight stearic acid, 15% by weight oleic acid and 1% 10 by weight linoleic acid. Rapeseed oil typically contains ca. 48% by weight erucic acid, 15% by weight oleic acid, 14% by weight linoleic acid, 8% by weight linolenic acid, 5% by weight eicosenoic acid, 3% by weight palmitic acid, 2% by weight hexadecenoic acid and 1% by weight docosadienoic acid as fatty acid components. Rapeseed oil from new plants is richer in 15 the unsaturated components. Typical fatty acid components here are erucic acid 0.5% by weight, oleic acid 63% by weight, linoleic acid 20% by weight, linolenic acid 9% by weight, eicosenoic acid 1% by weight, palmitic acid 4% by weight, hexadecenoic acid 2% by weight and docosadienoic acid 1% by weight. 80 to 85% by weight of castor oil consists of the 20 glyceride of ricinoleic acid. Castor oil also contains ca. 7% by weight glycerides of oleic acid, 3% by weight glycerides of linoleic acid and ca. 2% by weight glycerides of palmitic and stearic acid. 55 to 65% by weight of the total fatty acids in soybean oil are polyunsaturated acids, more particularly linoleic and linolenic acid. The situation with sunflower oil is 25 similar, its typical fatty acid spectrum - based on total fatty acid - being as follows: ca. 1% by weight myristic acid, 3 to 10% by weight palmitic acid, 14 to 65% by weight oleic acid and 20 to 75% by weight linoleic acid.

All the above numerical data on the fatty acid components of the triglycerides are dependent on the quality of the raw materials and, 30 accordingly, can vary. PIT emulsions containing group b) nutrients



heptadecanoic acid, stearic acid, nonadecanoic acid, arachic acid and behenic acid. Unsaturated representatives are, for example, lauroleic acid, myristoleic acid, palmitoleic acid, petroselaic acid, oleic acid, elaidic acid, ricinoleic acid, linoleic acid, linolaidic acid, linolenic acid, gadoleic acid, arachidonic acid and erucic acid. Mixtures of the methyl esters of these acids are also suitable. It is particularly preferred to use PIT emulsions containing methyl esters from the group consisting of methyl oleate, methyl palmitate, methyl stearate and/or methyl pelargonate. However, methyl esters based on natural fatty acid mixtures obtainable, for example, from linseed oil, coconut oil, palm oil, palm kernel oil, olive oil, castor oil, rapeseed oil, soybean oil or sunflower oil (in the case of rapeseed and sunflower oil, new and old plants) may also be used.

Suitable group b) compounds are native oils of vegetable origin. These are essentially triglyceride mixtures where the glycerol is always completely esterified with relatively long-chain fatty acids. Particularly suitable vegetable oils are selected from the group consisting of peanut oil, coconut oil and/or sunflower oil.

Peanut oil contains on average (based on fatty acid) 54% by weight oleic acid, 24% by weight linoleic acid, 1% by weight linolenic acid, 1% by weight arachic acid, 10% by weight palmitic acid and 4% by weight stearic acid and has a melting point of 2 to 3°C. Linseed oil typically contains 5% by weight palmitic acid, 4% by weight stearic acid, 22% by weight oleic acid, 17% by weight linoleic acid and 52% by weight linolenic acid and has an iodine value of 155 to 205, a saponification value of 188 to 196 and a melting point of about -20°C. Coconut oil contains ca. 0.2 to 1% by weight hexanoic acid, 5 to 8% by weight octanoic acid, 6 to 9% by weight decanoic acid, 45 to 51% by weight lauric acid, 16 to 19% by weight myristic acid, 9 to 11% by weight palmitic acid, 2 to 3% by weight stearic acid, less than 0.5% by weight behenic acid, 8 to 10% by weight oleic acid and up to 1% by weight linoleic acid as fatty acid components. It has an iodine value of 7.5

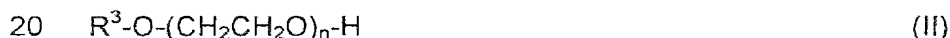
selected from coconut oil, sunflower oil and/or rapeseed oil are particularly preferred.

Important constituents of the PIT emulsions used in accordance with the invention are the emulsifiers and emulsifier systems used. Nonionic emulsifiers, more particularly ethoxylated fatty alcohols and fatty acids, are preferably used as emulsifiers. To form PIT emulsions, it is of advantage to use a two-component emulsifier system containing a hydrophilic emulsifier (A) and a hydrophobic co-emulsifier (B). Suitable hydrophilic nonionic emulsifiers (A) are substances which have an HLB value of about 8 to 18. The HLB value (hydrophilic/lipophilic balance) is a value which may be calculated in accordance with the following equation:

$$HLB = (100-L) / 5$$

where L is the percentage by weight of lipophilic groups, i.e. the fatty alkyl or fatty acyl groups in percent in the ethylene oxide addition products.

Fatty alcohol ethoxylates in the context of the teaching according to the invention correspond to formula (II):



in which  $R^3$  is a linear or branched, saturated or unsaturated alkyl group containing 6 to 24 carbon atoms and n is a number of 1 to 50. Compounds of formula (II) where n is a number of 1 to 35 and more particularly a number of 1 to 15 are particularly preferred. Other particularly preferred compounds of formula (II) are those where  $R^3$  is an alkyl group containing 16 to 22 carbon atoms.

The compounds of formula (II) are obtained in known manner by reaction of fatty alcohols under pressure with ethylene oxide, optionally in the presence of acidic or basic catalysts. Typical examples are caproic

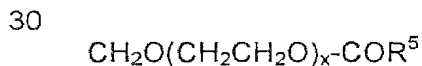
alcohol, caprylic alcohol, 2-ethyl hexyl alcohol, capric alcohol, lauryl alcohol, isotridecyl alcohol, myristyl alcohol, cetyl alcohol, palmitoleyl alcohol, stearyl alcohol, isostearyl alcohol, oleyl alcohol, elaidyl alcohol, petroselinyl alcohol, linolyl alcohol, linolenyl alcohol, elaeostearyl alcohol, arachyl alcohol, gadoleyl alcohol, behenyl alcohol, erucyl alcohol and brassidyl alcohol and the technical mixtures thereof obtained, for example, in the high-pressure hydrogenation of technical methyl esters based on fats and oils or aldehydes from Roelen's oxosynthesis and as monomer fraction in the dimerization of unsaturated fatty alcohols. Technical fatty alcohols containing 12 to 18 carbon atoms, such as for example coconut oil, palm oil, palm kernel oil or tallow fatty alcohol, are preferred.

Fatty acid ethoxylates which may also be used as emulsifier component (A) preferably correspond to formula (III):



where  $\text{R}^4$  is a linear or branched alkyl group containing 12 to 22 carbon atoms and  $m$  is a number of 5 to 50 and preferably 15 to 35. Typical examples are products of the addition of 20 to 30 moles ethylene oxide onto lauric acid, isotridecanoic acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, isostearic acid, oleic acid, elaidic acid, petroselic acid, linoleic acid, linolenic acid, elaeostearic acid, arachic acid, gadoleic acid, behenic acid and erucic acid and the technical mixtures thereof obtained for example in the pressure hydrolysis of natural fats and oils or in the reduction of aldehydes from Roelen's oxosynthesis. Products of the addition of 20 to 30 moles ethylene oxide onto  $\text{C}_{16-18}$  fatty acids are preferably used.

Partial glycerides which may be used as emulsifier component (B) preferably correspond to formula (IV):





5

where CO R<sup>5</sup> is a linear or branched acyl group containing 12 to 22 carbon atoms and x, y and z together stand for 0 or for numbers of 1 to 50 and preferably 15 to 35. Typical examples of partial glycerides suitable for the purposes of the invention are lauric acid monoglyceride, coconut fatty acid monoglyceride, palmitic acid monoglyceride, stearic acid monoglyceride, isostearic acid monoglyceride, oleic acid monoglyceride and tallow fatty acid monoglyceride and addition products thereof with 5 to 50 and preferably 20 to 30 moles ethylene oxide. Monoglycerides or technical mono/diglyceride mixtures predominantly containing monoglycerides (IV) where CO R<sup>5</sup> is a linear acyl group containing 16 to 18 carbon atoms, are preferably used.

Emulsifier mixtures containing components (A) and (B) in a ratio by weight of 10:90 to 90:10, preferably 25:75 to 75:25 and more particularly 40:60 to 60:40 are normally used.

Other suitable emulsifiers are, for example, nonionic surfactants from at least one of the following groups:

- (I) products of the addition of 2 to 30 moles of ethylene oxide and/or 0 to 5 moles of propylene oxide onto linear fatty alcohols containing 8 to 22 carbon atoms;
- (II) glycerol monoesters and diesters and sorbitan monoesters and diesters of saturated and unsaturated fatty acids containing 6 to 22 carbon atoms and ethylene oxide adducts thereof;
- (III) alkyl mono- and oligoglycosides containing 8 to 22 carbon atoms in the alkyl group and ethoxylated analogs thereof;
- (IV) products of the addition of 15 to 60 moles of ethylene oxide onto castor oil and/or hydrogenated castor oil;

- (V) polyol esters and, in particular, polyglycerol esters such as, for example, polyglycerol polyricinoleate or polyglycerol poly-12-hydroxystearate. Mixtures of compounds from several of these classes are also suitable;
- 5 (VI) products of the addition of 2 to 15 moles of ethylene oxide onto castor oil and/or hydrogenated castor oil;
- (VII) partial esters based on linear, branched, unsaturated or saturated  $C_{6/22}$  fatty acids, ricinoleic acid and 12-hydroxystearic acid and glycerol, polyglycerol, pentaerythritol, dipentaerythritol, sugar
- 10 alcohols (for example sorbitol) and polyglucosides (for example cellulose);
- (VIII) wool wax alcohols;
- (IX) polyalkylene glycols.

The addition products of ethylene oxide and/or propylene oxide onto

15 glycerol mono- and diesters and sorbitan mono- and diesters of fatty acids or onto castor oil are known commercially available products. They are homolog mixtures of which the average degree of alkoxylation corresponds to the ratio between the quantities of ethylene oxide and/or propylene oxide and substrate with which the addition reaction is carried out.

20 To select suitable emulsifier systems, it can be useful to calculate the PIT of the particular system. However, this also applies in particular to potential optimizations in the choice of the emulsifiers or emulsifier systems and their adaptation to the choice and mixing of aqueous phase on the one hand and the type of oil phase on the other hand as predetermined by

25 other considerations as to technical procedure. Corresponding expert knowledge has been developed in basically totally different fields, particularly in the production of cosmetics. Particular reference is made in this connection to the article by TH. Förster, W. von Rybinski, H. Tesmann and A. Wadle "Calculation of Optimum Emulsifier Mixtures for Phase

30 Inversion Emulsification" in International Journal of Cosmetic Science

**16, 84-92 (1994).** The article in question contains a detailed account of how the phase inversion temperature (PIT) range of a given three-component system of an oil phase, a water phase and an emulsifier can be calculated by the CAPICO method (calculation of phase inversion in concentrates) on the basis of the EACN value (equivalent alkane carbon number) characteristic of the oil phase. More particularly, this article by Förster et al. cites important literature for the subjects under discussion here which should be viewed in conjunction with the disclosure of this article by Förster et al. With the aid of numerous examples, it is shown how the choice and optimization of the emulsifiers/emulsifier systems are accessible to the adjustment of optimal predetermined values for the phase inversion temperature range by the CAPICO method in conjunction with the EACN concept.

The PIT emulsions used in accordance with the invention preferably contain 20 to 90% by weight of water, more preferably 30 to 80% by weight and most preferably 30 to 60% by weight of water. The balance to 100% by weight is made up of oil phase and emulsifiers and optionally other auxiliaries and additives. The oil phase itself is present in quantities of preferably 10 to 80% by weight and more particularly 40 to 70% by weight. In a preferred embodiment, the oil phase exclusively contains components a) or b) or mixtures of these components. The use of emulsions containing the oil and water phases in a ratio by weight of 1:1 is particularly preferred. The emulsifiers or emulsifier systems are present in quantities of preferably 1 to 25% by weight, more preferably 5 to 20% by weight and most preferably 5 to 15% by weight. The emulsions used in accordance with the invention preferably have phase inversion temperatures of 20 to 95°C and more particularly in the range from 40 to 95°C.

According to the invention, the described PIT emulsions may be used in fermentation processes of all kinds. Any of the various processes known to the expert, for example batch or fed batch and continuous

fermentation, may be used. In addition, any of the fermenter systems known to the expert may be used. For details, see *Crueger*, pages 50 to 70. Moreover, the use of the microemulsions is not confined to specific microorganisms. On the contrary, the emulsions may be used for the  
5 production or transformation of any of the compounds known to the expert through fermentation. Apart from the conventional fermentation processes which are mainly used for the synthesis of antibiotics (cf. *Crueger*, pages 197 to 242), the described emulsions are also suitable for use in microbial transformations (bioconversions), for example the transformation of  
10 steroids and sterols, antibiotics and pesticides or the production of vitamins (cf. *Crueger*, pages 254 to 273). However, the described emulsions are preferably used in fermentation processes for the production of antibiotics, for example cephalosporins, tylosine or erythromycin.

In general, the emulsions are suitably added to the aqueous  
15 fermentation broth containing the microorganisms and the nitrogen source and trace elements and optionally other auxiliaries, especially defoamers. Suitable nitrogen sources are, for example, peptone, yeast or malt extract, corn steep liquor, urea or lecithins. The trace elements may be present in the form of inorganic salts, for example sodium or potassium nitrate,  
20 ammonium nitrate, ammonium sulfate, iron sulfate, etc. It can also be of advantage to add other additives, such as defoamers or nitrogen sources, to the PIT emulsions themselves.

### Examples

Various emulsions were prepared by mixing the starting materials, heating the mixture beyond the PIT temperature and then cooling it to room temperature (20°C) (method according to DE 38 19 193 A1). The PIT temperature was determined by conductivity measurement. The droplet size was measured with a Coulter N4 Plus Submicron Particle Sizer. The measuring angle was 90°. The results are set out in Tables 1a and 1b.

The emulsions are suitable, for example, as sole nutrient source for fermentation processes and may be directly added to the aqueous fermentation broth.

Table 1a

	wt. %	wt. %	wt. %	wt. %	wt. %	wt. %	wt. %	wt. %
Rapeseed oil	34	45	45	45	45	40	40	45
Water	55	37	37	37	37	44	44	37
Castor oil ethoxylated with 40 moles EO per mole castor oil	5	3						
Behenyl alcohol + 10 EO	2.3		3	5	4	3	3	3
Glycerol oleate	3.7			2	1		1	
Hydrogenated castor oil ethoxylated with 7 moles EO per mole castor oil		15	15	11	13	13	12	13
PIT (°C)	84	83	68	61	65	72	57	75
Droplet size (nm)	318	293	230	187	195	229	175	269

Table 1b

	wt. %	wt. %	wt. %	wt. %	wt. %
Methyl oleate	45	45			
Sunflower oil				45	40
Coconut oil			34		
Water	45	45	55	37	44
C <sub>16/18</sub> fatty alcohol + 12 EO	5	7			
Castor oil ethoxylated with 40 moles EO per mole castor oil			4		
Behenyl alcohol + 10 EO			2.8	3	3
Glycerol monostearate		3			
Glycerol oleate			4.2		
Hydrogenated castor oil ethoxylated with 7 moles EO per mole castor oil				15	12
C <sub>16</sub> fatty alcohol + 6 EO	5				
PIT (°C)	73	74	62	68	57
Droplet size (nm)	201	216	156	219	180

## CLAIMS

1. The use of o/w emulsions containing at least water, emulsifiers and



an oil phase containing one or more compounds selected from the groups of

- a) fatty acid alkyl esters and/or
- b) triglycerides of vegetable origin

5

characterized in that the emulsion is produced by the PIT method and has a droplet size of 50 to 400 nm, in fermentation processes.

2. The use claimed in claim 1, characterized in that the oil phase  
10 contains fatty acid methyl esters as component a).

3. The use claimed in claim 1 or 2, characterized in that emulsions with a mean droplet size of 100 to 300 nm, preferably 180 to 300 nm and more particularly 160 to 250 nm are used.

4. The use claimed in claims 1 to 3, characterized in that emulsions  
15 containing water in quantities of 20 to 90% by weight, preferably 30 to 80% by weight and more particularly 30 to 60% by weight are used.

5. The use claimed in claims 1 to 4, characterized in that emulsions containing the oil phase in quantities of 10 to 80% by weight and preferably 40 to 70% by weight are used.

20 6. The use claimed in claims 1 to 5, characterized in that emulsions containing fatty acid methyl esters of formula (I):



25 in which  $R^1$  is a  $C_{6-22}$  alkyl group and  $R^2$  is a methyl group, are used.

7. The use claimed in claims 1 to 6, characterized in that emulsions containing methyl oleate, methyl palmitate, methyl stearate and/or methyl pelargonate in the oil phase are used.

30 8. The use claimed in claims 1 to 7, characterized in that emulsions

containing coconut oil, sunflower oil and/or rapeseed oil in the oil phase are used.

9. The use claimed in claims 1 to 8, characterized in that emulsions containing an emulsifier system containing hydrophilic emulsifiers with HLB  
5 values of 8 to 18 in combination with hydrophobic co-emulsifiers are used

10. The use claimed in claim 9, characterized in that emulsions of which the emulsifier systems have quantity ratios between hydrophilic emulsifiers and co-emulsifiers of 10:90 to 90:10 are used.

11. The use claimed in claims 1 to 10, characterized in that emulsions  
10 containing emulsifiers in quantities of 1 to 25% by weight, preferably in quantities of 5 to 20% by weight and more particularly in quantities of 5 to 15% by weight are used.

(19)



JAPANESE PATENT OFFICE

## PATENT ABSTRACTS OF JAPAN

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**KAMIYA SHUNICHI**

(54) **METHOD FOR PRODUCING**  
**TETRAHYDROCUCUMIN**

(57) Abstract:

PROBLEM TO BE SOLVED: To provide a method for producing tetrahydrocurcumins by using a microbial fungus, a cultured product or a treated product thereof, and to provide a method for producing a composition containing the tetrahydrocurcumins.

SOLUTION: In this method for producing the tetrahydrocurcumins or the composition containing the tetrahydrocurcumins, the microbial fungus, the cultured product or the treated product thereof, having activities for converting curcumins into the tetrahydrocurcumins is subjected to act on the curcumins or the composition containing the curcumins in the presence of an O/W type emulsion.

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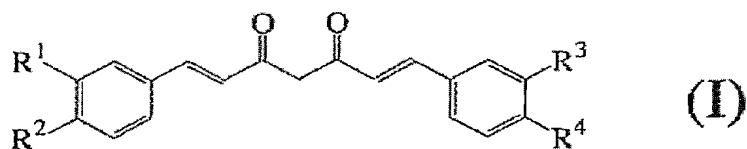
CLAIMS

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[Claim(s)]

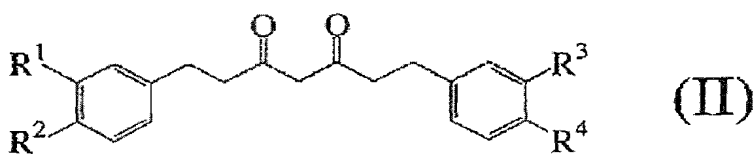
[Claim 1]The bottom of existence of O/W emulsion, formula (I)

[Formula 1]



[— formula (I) — the curcumin or the curcumin derivative (only henceforth curcumin) which R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> are the same or different inside, and is shown by] to which hydrogen and hydroxy \*\* express low-grade ARUKOKISHI — formula (II)

[Formula 2]



R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> among [type (II), The tetrahydro curcumin or the tetrahydro curcumin derivative shown by] to which it is the same or different and hydrogen and hydroxy \*\* express low-grade ARUKOKISHI. The biomass of the microorganism which has the activity changed for (only calling it tetrahydro curcumin hereafter), A manufacturing method of the constituent containing the tetrahydro curcumin or tetrahydro curcumin making culture medium or those treatment objects act on the

constituent containing curcumin or curcumin.

[Claim 2]The manufacturing method according to claim 1 with which a constituent containing curcumin or curcumin is included by O/W emulsion.

[Claim 3]The manufacturing method according to claim 1 or 2 which is what is produced by O/W emulsion mixing an oil, an emulsifier or a surface-active agent, and an aqueous medium.

[Claim 4]A way according to any one of claims 1 to 3 mean particle diameter of O/W emulsion is 10 nm – 10 micrometers.

[Claim 5]a constituent containing curcumin -- curcuma rhizoma -- a method according to any one of claims 1 to 4 of being a constituent produced by processing vegetation belonging to a group.

[Claim 6]A way according to any one of claims 1 to 5 a microorganism is a microorganism belonging to a DEBARYOMYCES group, Saccharomyces, Pichia, the Kluyveromyces group, the genus Torulaspora, the Candida group, the Lactobacillus group, a Staphylococcus group, or a PEJIOKOKKASU group.

[Claim 7]A biomass of a microorganism which has the activity which changes curcumin into tetrahydro curcumin under oil and an emulsifier, or surface-active agent existence among an aqueous medium, A manufacturing method of a constituent containing the tetrahydro curcumin or tetrahydro curcumin making culture medium or those treatment objects act on a constituent containing curcumin or curcumin.

[Claim 8]a constituent containing curcumin -- curcuma rhizoma -- a method according to claim 7 of being a constituent produced by processing vegetation belonging to a group.

[Claim 9]A way according to claim 7 or 8 a microorganism is a microorganism belonging to a DEBARYOMYCES group, Saccharomyces, Pichia, the Kluyveromyces group, the genus Torulaspora, the Candida group, the Lactobacillus group, a Staphylococcus group, or a PEJIOKOKKASU group.

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DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[Field of the Invention]This invention relates to the manufacturing method of the constituent containing tetrahydro curcumin or tetrahydro curcumin.

[0002]

[Description of the Prior Art]curcumin -- the tropical department of ginger -- curcmae rhizoma -- it is well known as yellow coloring matter contained in the vegetation belonging to a group. The curcumin which is the dried powder end of the rhizome of curcmae rhizoma (turmeric) and its refined material is used as flavors and the coloring agent of foodstuffs. It is known that curcumin has drug effect, such as an antioxidant action, anti-inflammatory activity, a cholesterol reduction operation, and a cancer inhibition operation.

It is used as safe medicinal properties of foodstuffs origin.

[0003][JP,H2-49747,A by which it is known that tetrahydro curcumin (THU1) has stronger antioxidation activity as compared with curcumin, JP,H2-51595,A, bioscience biotechnology and biochemistry (Biosci. Biotech. Biochem.), 59, 1609(1995)]. Since tetrahydro curcumin is colorlessness and odorlessness, when especially coloring serves as evil, it serves as a raw material which conquered the fault of curcumin.

[0004]The manufacturing method by the hydrogenation of the curcumin using the metal catalyst as a manufacturing method of tetrahydro curcumin is known. However, using the synthetic tetrahydro curcumin manufactured by this method for a food-and-drinks use has a food-sanitation-hygine top problem. Although the

manufacturing method (JP,H11-235192,A) of the tetrahydro curcumin by mixing the biomass etc. and curcumin of a microorganism to edible and keeping it warm as a suitable method of manufacturing available tetrahydro curcumin to it is raised, improvement in the further yield is called for.

[0005]The solubility to the water of the curcumin which is typical curcumin is low less than in ml and 0.1 microg /at ordinary temperature. When performing the biochemical reaction which uses a microbial cell etc. by a drainage system, with the organic solvent which dissolves curcumin, such as ethanol, dissolve in water first, and use poorly soluble curcumin for it, but, It is difficult for a microbial cell etc. to inactivate, if curcumin precipitates or the amount of organic solvents to be used is raised into reaction mixture, and to make it react efficiently. An organic solvent having some which show toxicity and using a resultant in the food-and-drinks field may be restricted.

[0006]An organic solvent cannot be used, but water can be made to distribute poorly soluble curcumin well to a drainage system, and art which makes recovery of an object easy [ balance / of the generation reaction and decomposition reaction of an object / former ] further is desired. Emulsion-ized art is the method of using well in food and drinks and the drugs field in order to make water distribute the compound of damage-at-sea solubility, but. About using for a structure ornamentation reaction, if the combination of an enzyme and output shows, Alkaline phosphatase and PARANITRO phenyl phosphate (p-nitrophenylphosphate) [Indian Journal of Biochemistry& Biophysics, 32, and 261] (1995), Lipase and 16-hydroxyhexadecanoic acid (16-hydroxyhexadecanoic acid) [Biochemica et Biophysica Acta, 1257, and 239 (1995)], Bilirubin oxidase and bilirubin [European Journal of Biochemistry, 183, and 347] (1989), And although there is a report about phospholipase A2 and phosphatidylcholine [Colloids and Surfaces, 128, and 17] (1997), there is no report used for the structural transition reaction of the curcumin using a microbial cell etc.

[0007]

[Problem(s) to be Solved by the Invention]The purpose of this invention is to provide the manufacturing method of the constituent containing the manufacturing method of tetrahydro curcumin and tetrahydro curcumin which used a microbial cell, cultures, or those treatment objects.

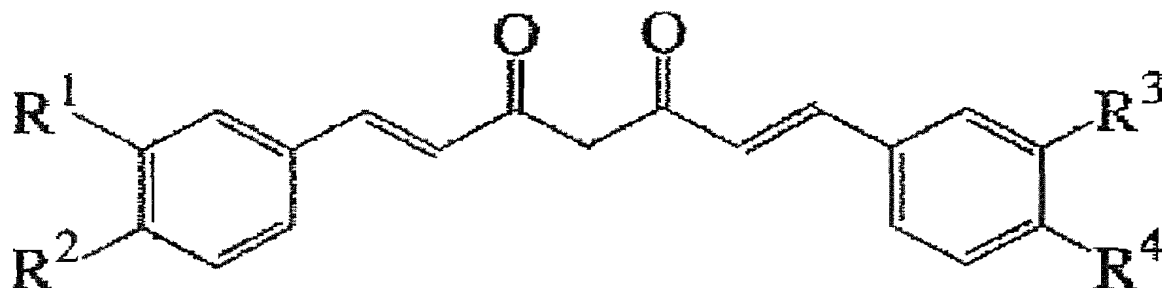
[0008]

[Means for Solving the Problem]This invention relates to the following (1) - (9).

(1) The bottom of existence of O/W emulsion, formula (I)

[0009]

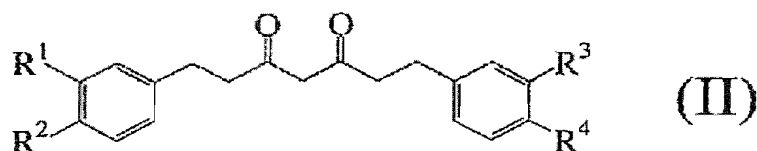
[Formula 3]



[0010][-- formula (I) -- the curcumin or the curcumin derivative (only henceforth curcumin) which  $R^1$ ,  $R^2$ ,  $R^3$ , and  $R^4$  are the same or different inside, and is shown by] to which hydrogen and hydroxy \*\* express low-grade ARUKOKISHI -- formula (II)

[0011]

[Formula 4]



[0012] $R^1$ ,  $R^2$ ,  $R^3$ , and  $R^4$  among [type (II), The tetrahydro curcumin or the tetrahydro curcumin derivative shown by] to which it is the same or different and hydrogen and hydroxy \*\* express low-grade ARUKOKISHI. The biomass of the microorganism which has the activity changed for (only calling it tetrahydro curcumin hereafter), A manufacturing method of the constituent containing the tetrahydro curcumin or tetrahydro curcumin making culture medium or those treatment objects act on the constituent containing curcumin or curcumin.

[0013](2) A manufacturing method given in (1) with which a constituent containing curcumin or curcumin is included by O/W emulsion.

(3) A manufacturing method given in (1) which is what is produced by O/W emulsion mixing an oil, an emulsifier or a surface-active agent, and an aqueous medium, or (2).

(4) A method given in either of (1) - (3) whose mean particle diameter of O/W emulsion is 10 nm - 10 micrometers.

[0014](5) a constituent containing curcumin -- curcuma rhizoma -- a method given in either of (1) - (4) which is a constituent produced by processing vegetation belonging to a group.



(6) A method given in either of (1) – (5) whose microorganisms are microorganisms belonging to a DEBARYOMAISESU group, Saccharomyces, Pichia, the Kluyveromyces group, the genus Torulaspora, the Candida group, the Lactobacillus group, a Staphylococcus group, or a PEJIOKOKKASU group.

[0015](7) Under oil and an emulsifier, or surface-active agent existence among an aqueous medium, A biomass of a microorganism which has the activity which changes curcumin into tetrahydro curcumin, A manufacturing method of a constituent containing the tetrahydro curcumin or tetrahydro curcumin making culture medium or those treatment objects act on a constituent containing curcumin or curcumin.

[0016](8) a constituent containing curcumin — curcuma rhizoma — a method given in (7) which is a constituent produced by processing vegetation belonging to a group.

(9) A method given in (7) whose a microorganism is a microorganism belonging to a DEBARYOMAISESU group, Saccharomyces, Pichia, the Kluyveromyces group, the genus Torulaspora, the Candida group, the Lactobacillus group, a Staphylococcus group, or a PEJIOKOKKASU group, or (8).

[0017]

[Embodiment of the Invention]The alkyl part of low-grade ARUKOKISHI of R<sup>1</sup> in formula (I) and formula (II), R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup>, It is a straight chain of the carbon numbers 1–6, or the alkyl of branching, for example, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, pentyl, hexyl, etc. are raised. If alkoxy \*\* is carried out, the methoxy of the carbon numbers 1–2 and ETOKISHI are preferred. In formula (I), a hydroxy \*\*\*\*\* compound is preferred as R<sup>2</sup> and R<sup>4</sup>.

[0018]As concrete curcumin, it is U1[JIFERU roil methane (], for example.

[ diferuloylmethane and ] (E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene 3,5-dione, ] which may only be called “curcumin” below, and U2[DIME — an ibis — Sickie cumin (demethoxycurcumin). 4-hydroxycinnamoyl (feruloyl) methane], U3[BISUDI methoxycurcumin (bisdemethoxycurcumin), bis(4-hydroxycinnamoyl) methane], DMU1 [(E, E)-1,7-bis(3, 4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione], DHU1 [(E, E)-1,7-Bis(3, 4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione] etc. is raised. U1, U2, U3, and DHU1 are used preferably, and U1 and DHU1 are used especially preferably.

[0019]Although the manufacturing method of the O/W emulsion used for this invention does not have restriction in particular, oil, an emulsifier or a surface-active agent, and an aqueous medium can be mixed and manufactured, for example. Although there is no restriction in particular as the mixing ratio, 0.01 to emulsifier or surface-active agent 10 weight section and one to aqueous medium 100 weight

section are preferred to oil 1 weight section, for example, and 0.1 to emulsifier or surface-active agent 1 weight section and two to aqueous medium 50 weight section are more preferred.

[0020]The preparing method of an emulsion does not have restriction in particular, and Agitators, such as a propeller mixer and a turbine mixer, A high velocity revolution rotor disk type emulsion machine like a colloid mill, a high velocity revolution type distribution emulsion machine like a homomixer, A high voltage high speed injection type emulsion machine like a jet homogenizer, an ultrasonic wave, a high voltage homogenizer, a vortex mixer, a magnetic stirrer, and the method of preparing using a high voltage homogenizer preferably are raised.

[0021]As a manufacturing method of O/W emulsion, for example, 0.1 to fats-and-oils 1 weight section, 0.1 to emulsifier 1 weight section, and one to aqueous medium 10 weight section are mixed, and the expenses [ rpm / 1,000-20,000 ] of the method of stirring for 1 to 10 minutes are covered by a mixer, keeping it warm at about 40-60 \*\*. Although the mean particle diameter of O/W emulsion does not have restriction in particular, 10 nm - 10 micrometers are preferred, 50 nm - 5 micrometers are more preferred, and 100 nm - especially 2 micrometers are preferred.

[0022]The constituent containing curcumin or curcumin may be included as O/W emulsion used for this invention. As the concrete method of preparation of the O/W emulsion which includes the constituent containing curcumin or curcumin, for example, curcumin 1 weight section -- oil -- two to 1000 weight section, it adds to five to 100 weight section preferably, and heating fusion is preferably carried out at 50-200 \*\*, and 0 \*\* - 300 \*\* of curcumin content oil is created. The O/W emulsion which includes the constituent which processes like the above-mentioned emulsion preparing method using this curcumin content oil, and contains curcumin or curcumin can be manufactured.

[0023]As oil, especially if curcumin can be dissolved, there will be no restriction, For example, soybean oil, sesame oil, a safflower, cottonseed cake oil, coconut oil, coconut oil, Vegetable oil, such as diacylglycerol content vegetable oil, long-chain-fatty-acid triglyceride or medium-chain-fatty-acid triglyceride (MCT), the alpha-tocopherol, saturated fatty acid, unsaturated fatty acid, cholesterol, etc. are raised.

[0024]As an emulsifier or a surface-active agent, a lecithin system emulsifier, a saponin system emulsifier, a polysaccharide system emulsifier, a protein system emulsifier, other emulsifiers, a synthetic surfactant, etc. are raised, for example. As a lecithin system emulsifier, enzymatically decomposed lecithin, such as enzymatically

modified lecithin, such as vegetable lecithin, such as yolk lecithin and soybean origin lecithin, and a phosphatidylglycerol, lysolecithin, and phosphatidic acid, etc. are raised, for example.

[0025]As a saponin system emulsifier, quillaja saponin, enju saponin, soybean saponin, a yucca home, etc. are raised, for example. As a polysaccharide system emulsifier, modified starch, such as gum arabic, a carrageenan, xanthan gum, and octenylsuccinic acid starch, alginic acid propylene glycerol ester, etc. are raised, for example.

[0026]Soybean protein etc. are raised as a protein system emulsifier in for example, a gluten partial decomposition product, a plant protein partial decomposition product, gliadin, casein, casein sodium, milk serum protein, casein, and the end of albumen powder. As other emulsifiers, sphingolipid, plant sterol, zoosterol, powdered bile, tomato glucolipid, etc. are raised, for example.

[0027]as a synthetic surfactant -- for example, a polyether type polymer surfactant and a glycerine fatty acid ester (a glycerine fatty acid ester.) Glycerin acetate ester, glycerin acetic acid fatty acid ester, glycerin lactic acid fatty acid ester, Glycerin citrate fatty acid ester, glycerin succinic acid fatty acid ester, Glycerin diacetylacid tartaric acid fatty acid ester, polyglyceryl fatty acid ester, etc., Sucrose fatty acid ester, a sorbitan fatty acid ester, propylene glycol fatty acid ester, calcium stearoyl lactylate, oxyethylene higher-fatty-acid alcohol, sodium oleate, morpholine fatty acid salts, polyoxyethylene higher aliphatic alcohol, etc. can raise, and it is \*\*\*.

[0028]As an aquosity medium, glycerol, pure water, buffer solution, etc. are used, for example. As buffer solution, the buffer solution of Clark-Lubs, the buffer solution of Sorensen, The buffer solution of Kolthoff, buffer solution of Michaelis, buffer solution of Atkins-Pantin, The buffer solution of Palitzsch, the buffer solution of McIlvaine, the buffer solution of Menzel, the buffer solution of Walpole, the buffer solution of Hasting-Sendroy, the buffer solution of Gomori, etc. are raised.

[0029]As buffer solution of Clark-Lubs, potassium chloride chloride, acid-potassium-phthalate chloride, acid-potassium-phthalate sodium hydroxide, potassium-dihydrogen-phosphate sodium hydroxide, borate salt-ized potassium sodium hydroxide, etc. are raised, for example. As buffer solution of Sorensen, for example Glycine sodium salt acid chloride, Glycine sodium sodium hydroxide chloride, sodium-acid-citrate chloride, sodium-acid-citrate sodium hydroxide, borax-chloride, borax-sodium hydroxide, phosphoric acid 2 hydrogen potassium hydrogen phosphate NIKARIUMU, etc. are raised.

[0030]As buffer solution of Kolthoff, for example Aqueous-citric-acid matter potassium citrate, Aqueous-citric-acid matter potassium salt acid,

aqueous-citric-acid matter potassium sodium hydroxide, succinic acid-borax, aqueous-citric-acid matter potassium borax, phosphoric acid 2 hydrogen potassium borax, and borax-sodium carbonate, chloride-sodium carbonate, disodium hydrogenphosphate sodium hydroxide, etc. are raised.

[0031]As buffer solution of Michaelis, for example The tartaric acid-sodium tartrate, Lactic acid-sodium lactate, acetic acid-sodium acetate, phosphoric acid 2 hydrogen potassium disodium hydrogenphosphate, An ammonium chloride ammonia solution, sodium diethylbarbiturate acetic acid sodium salt acid, sodium diethylbarbiturate chloride, dimethylglycine sodium salt acid, etc. are raised.

[0032]As buffer solution of Atkins-Pantin, for example, borate salt-ized potassium sodium carbonate etc. are raised. As buffer solution of Palitzsch, a borate salt-ized sodium borax etc. are raised, for example. As buffer solution of McIlvaine, for example, hydrogen phosphate disodium citrate etc. are raised.

[0033]As buffer solution of Menzel, sodium carbonate sodium bicarbonate etc. are raised, for example. As buffer solution of Walpole, sodium acetate chloride and acetic acid-sodium acetate etc. are raised, for example. As buffer solution of Hasting-Sendroy, hydrogen phosphate disodium potassium dihydrogen phosphate etc. are raised, for example.

[0034]As buffer solution of Gomori, tris buffers, such as 2, 4,6-trimethyl pyridine-chloride, and tris aminomethane chloride, 2-aminomethyl 1,3-propanediol chloride, etc. are raised, for example. In this invention, as for oil, the emulsifier or \*\*\*\*\*, and the aquosity medium which are used for preparation of O/W emulsion, it is preferred that it is a thing of natural origin, and it is preferred that it is the ingredient approved by edible.

[0035]As oil used especially for preparation of O/W emulsion, Medium-chain-fatty-acid triglyceride (MCT) is preferred, it is preferred as an emulsifier or a surface-active agent to use the mixture of polyglyceryl fatty acid ester and enzymatically decomposed lecithin, and it is preferred as an aquosity medium to use pure water. The manufacturing method of the constituent containing the tetrahydro curcumin or tetrahydro curcumin of this invention, The process which makes the biomass, the culture medium, or those treatment objects of the microorganism which has the activity which changes curcumin into tetrahydro curcumin act on the constituent containing curcumin or curcumin is included under existence of O/W emulsion.

[0036]The manufacturing method of the constituent containing the tetrahydro curcumin or tetrahydro curcumin of this invention, The process which makes the

biomass, the culture medium, or those treatment objects of the microorganism which has the activity which changes curcumin into tetrahydro curcumin under oil and an emulsifier, or surface-active agent existence act on the constituent containing curcumin or curcumin is included among an aqueous medium.

[0037]In addition to aqueous media, such as water, reaction mixture is prepared if needed in the O/W emulsion prepared by the constituent and the above-mentioned method containing the culture medium, the biomasses or those treatment objects, curcumin, or curcumin of this microorganism. As reaction mixture, the constituent, oil and emulsifier, or surface-active agent which contains the biomass, the culture medium or those treatment objects, curcumin, or curcumin of this microorganism to an aqueous medium is added, and it is prepared.

[0038]Manufacture of the constituent containing tetrahydro curcumin or tetrahydro curcumin, this reaction mixture -- for example, 0-100 \*\* -- desirable -- 10-60 \*\* -- especially -- desirable -- 22-45 \*\* and pH -- 2-11 -- desirable -- 3-9 -- it can carry out by keeping it warm preferably for 0.5 to 72 hours under the conditions of 4-8 preferably especially for 0.01 to 168 hours.

[0039]Especially if it has the activity which changes curcumin into tetrahydro curcumin as a quantity of the biomass of the microorganism in reaction mixture, culture medium, or these treatment objects, will not be limited, but. For example, it adds to reaction mixture so that 1micro g-800mg/ml may become in ml and 10-500mg /preferably by wet fungus body weight. Although the constituent concentration in particular containing the curcumin or curcumin in reaction mixture does not have restriction, it adds so that 0.001-500mg/ml may come in ml and 0.01-100mg /preferably as curcumin, for example into reaction mixture.

[0040]Although there is no restriction in particular, the O/W emulsion concentration in reaction mixture is added so that 0.01-990mg/ml may become in ml and 1-100mg /preferably, for example. Although the concentration in particular of oil in reaction mixture does not have restriction, it adds so that 0.01-990mg/ml may become in ml and 1-100mg /preferably, for example. Although the concentration in particular of the emulsifier in reaction mixture or a surface-active agent does not have restriction, it adds so that 0.01-990mg/ml may become in ml and 1-100mg /preferably, for example.

[0041]As reaction mixture, the biomass, the culture medium, or these treatment objects of a microorganism, for example 0.5 to 1000 weight section, What one to 10 weight section and O/W emulsion become from 0.05 to 10 weight section preferably 0.0001 to 1000 weight section 0.5 to 1000 weight section in the constituent which contains one to 10 weight section, curcumin, or curcumin preferably is raised.

[0042]When the constituent containing curcumin or curcumin is included by O/W emulsion, Manufacture of the constituent containing tetrahydro curcumin or tetrahydro curcumin, The process made to act on the O/W emulsion which includes the constituent which contains curcumin or curcumin for the biomass, the culture medium, or those treatment objects of the microorganism which has the activity which changes curcumin into tetrahydro curcumin is included.

[0043]In addition to aqueous media, such as water, the O/W emulsion which includes the constituent containing the biomass, the culture medium or those treatment objects and curcumin, or curcumin of this microorganism is made into reaction mixture if needed, this reaction mixture -- 0-100 \*\* -- desirable -- 10-60 \*\* -- especially -- desirable -- 20-50 \*\* and pH -- 2-11 -- desirable -- 3-9 -- it is made to react preferably under the conditions of 4-8 especially for 0.5 to 72 hours for 0.01 to 168 hours

[0044]Especially if it has the activity which changes curcumin into tetrahydro curcumin as a quantity of the biomass of the microorganism in reaction mixture, culture medium, or these treatment objects, will not be limited, but. For example, it adds to reaction mixture so that 1micro g-800mg/ml may become in ml and 10-500mg /preferably by wet fungus body weight. Although there is no restriction in particular, the O/W emulsion concentration containing the constituent containing the curcumin or curcumin in reaction mixture is added so that it may become in ml and 1-100mg /preferably [ ml ] 0.01-990mg /, for example.

[0045]Although the constituent concentration in particular containing the curcumin or curcumin in this O/W emulsion does not have restriction, it is preferred to use the emulsion which is [ ml ] 1-200mg/ml preferably 0.01-999mg /as for example, curcumin into this emulsion. The biomass, the culture medium, or these treatment objects of a microorganism reaction mixture, for example 0.5 to 1000 weight section, In the constituent O/W emulsion which contains one to 10 weight section and curcumin, or curcumin preferably, 0.05 to 10 weight section and an aqueous medium consist of ten to 100 weight section preferably zero to 1000 weight section 0.0001 to 1000 weight section.

[0046]Buffer solution, an emulsifier or a surface-active agent, an organic solvent, a coenzyme, etc. can be made to exist if needed in reaction mixture. As buffer solution, other dibasic sodium phosphate of the above-mentioned buffer solution, a glycine, N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid, ammonium acetate, a tris(hydroxymethyl) methylglycine, etc. are raised.

[0047]As an emulsifier or a surface-active agent, triton X100 grade besides the

above-mentioned emulsifier or a surface-active agent is raised. Ethanol, methanol, glycerin, etc. are raised as an organic solvent. As a coenzyme, for example, beta nicotinamide adenine dinucleotide, beta nicotinamide adenine dinucleotide phosphate, reduction type beta nicotinamide adenine dinucleotide, reduction type beta nicotinamide adenine dinucleotide phosphate, etc. are raised.

[0048]It may refine and the constituent containing the tetrahydro curcumin or tetrahydro curcumin generated in reaction mixture may be used, although it can use as food and drinks or feed as it is. Although there is no restriction in particular as a refining method, organic solvent extraction, a centrifuge method, column chromatography, a freeze drying method, recrystallizing method, a hot-air-drying method, etc. are raised, for example.

[0049]Curcumin and tetrahydro curcumin output can be analyzed and detected by HPLC etc. Although the reaction of the biomass of the constituent containing curcumin or curcumin and a microorganism, culture medium, or those treatment objects can also be performed by carrying out fixed time neglect using containers, such as wooden slack, without carrying out control of temperature, quantity of airflow, and a hydrogen-ion density, for example, It is preferred to carry out using the fermentation device (jar fermenter) which is automatic or semiautomatic and can perform control of temperature, quantity of airflow, and a hydrogen-ion density.

[0050]In being a microorganism which has the alcoholic fermentation or lactic-acid-fermentation ability other than activity from which the microorganism to be used changes curcumin into tetrahydro curcumin, By adding the constituent containing the O/W emulsion, curcumin, or curcumin which includes the constituent containing curcumin or curcumin in the middle of alcoholic fermentation or lactic acid fermentation, and the O/W emulsion which does not include them, Or by adding the constituent, oil and emulsifier, or surface-active agent containing curcumin or curcumin, the alcoholic beverage or lactic fermentation food containing tetrahydro curcumin can also be manufactured.

[0051]The curcumin content in the constituent containing the curcumin used in order to manufacture the constituent which contains tetrahydro curcumin by this invention is 0.1% to 99.9% preferably 99.999% from 0.01%. Although there is no restriction in the total capacity of reaction mixture, 1000 kl is 1000 kl from 1 ml preferably from 0.1 ml. The residue of the curcumin in the reaction mixture after a reaction is 99% to 0.001% to the curcumin before a reaction, and is 95% to 0.001% preferably. 1 mg - 1 g per g of curcumin before a reaction of generated amounts of the tetrahydro curcumin after a reaction are 10 mg - 1g preferably.

[0052]Tetrahydro curcumin arises in the constituent which contains the curcumin which do not contain tetrahydro curcumin substantially by this invention, and the constituent containing tetrahydro curcumin can be obtained. By this invention, the content of the tetrahydro curcumin in the constituent containing both tetrahydro curcumin and curcumin can be raised, and the yellow of the curcumin in this constituent can be reduced.

[0053]Although pure tetrahydro curcumin can be substantially manufactured by this invention, Reaction mixture can be used as it is as foodstuffs, an alcoholic beverage, a food additive, medicine, animal feed, fishery feed, an animal drug, an antioxidant, cosmetics, or those raw materials, without separating the biomass, the culture medium, or these treatment objects of the microorganism used for the reaction from reaction mixture.

[0054]a turmeric -- curcmae rhizoma -- it is in the dried powder end of the rhizome of the vegetation belonging to a group, and they are foodstuffs used as a raw material of turmeric tea or spices. The curcumin refined from a turmeric is the substances currently widely used for food manufacturing as safe edible natural coloring matter. therefore -- in this invention -- curcmae rhizoma -- by using the constituent containing the curcumin refined from the debris, the extract, fraction, or turmeric of the vegetation belonging to a group as a constituent containing curcumin, The constituent containing suitable tetrahydro curcumin with very high safety to use as foodstuffs, an alcoholic beverage, a food additive, medicine, animal feed, fishery feed, an animal drug, an antioxidant, cosmetics, and those raw materials can be obtained.

[0055]Various constituents which contain tetrahydro curcumin by the method of this invention, for example, foodstuffs, an alcoholic beverage, a food additive, medicine, animal feed, fishery feed, an animal drug, an antioxidant, cosmetics, and those raw materials can be manufactured. Although the range of this invention is not limited with the manufacturing method hung up over below, For example, manufacture of the turmeric tea containing the tetrahydro curcumin the debris and yeast of a turmeric are made to act on, Manufacture of the curry powder containing the tetrahydro curcumin a turmeric and yeast are made to act on, Manufacture of the yeast foodstuffs containing the tetrahydro curcumin a turmeric and yeast are made to act on, Yeast containing the tetrahydro curcumin obtained by making curcumin and yeast act Butter, Manufacture etc. of the alcoholic beverage containing the tetrahydro curcumin obtained by making the manufacture and the turmeric, and yeast of tetrahydro curcumin content cooking oil which immerse in cooking oil, such as vegetable oil or sesame oil, act are raised.



[0056]The turmeric tea containing tetrahydro curcumin can be manufactured, for example under existence of O/W emulsion by making the biomass, the culture medium, or those treatment objects of the microorganism which has the activity which changes curcumin into tetrahydro curcumin act on the debris of a turmeric.

[0057]The curry powder containing tetrahydro curcumin, For example, the biomass of the microorganism which has the activity which changes curcumin into tetrahydro curcumin under existence of O/W emulsion, By using the constituent containing the tetrahydro curcumin which obtained them by making culture medium or those treatment objects act on the constituent containing curcumin or a turmeric as a raw material of curry powder, The curry powder which raised the antioxidant function originating in the curcumin in curry powder and the medicinal value can be manufactured. Namely, what is necessary is just to use the constituent obtained by this invention as substitution of a turmeric by the manufacturing process of the curry powder which mixes various spices. Since the yellow of curry powder is what is depended on the curcumin contained in the turmeric of a curry powder raw material, If it is going to raise the amount of turmeric in curry powder, or the quantity of curcumin in order to manufacture the curry powder which heightened efficacy other than coloring or aromatizing among the efficacy which curcumin has, it is not practical in order to bring about the coloring and aromatizing more than needed. However, when the curcumin or the turmeric which raised the content of tetrahydro curcumin by this invention is used for the raw material of curry powder. Since yellow has decreased according to the content of colorless, odorless tetrahydro curcumin, the function which tetrahydro curcumin has can be given without exceeding the range of the degree of coloring permitted as curry powder, or fragrance. However, as long as it is within the limits permitted as edible, it is not as substitution of a turmeric and the tetrahydro curcumin obtained by the presentation of curry powder by the invention may be added.

[0058]The yeast foodstuffs containing tetrahydro curcumin, For example, after making the food yeast which has the activity which changes curcumin into tetrahydro curcumin under existence of O/W emulsion act on the constituent containing curcumin or a turmeric and making tetrahydro curcumin generate, It can manufacture by collecting the whole reactants by freeze-drying or hot air drying. The new yeast foodstuffs which gave the function of tetrahydro curcumin to the yeast foodstuffs currently conventionally manufactured aimed at obtaining nourishment can be obtained.

[0059]The yeast foodstuffs containing tetrahydro curcumin, For example, after making

the food yeast which has the activity which changes curcumin into tetrahydro curcumin under existence of O/W emulsion act on the constituent containing curcumin or a turmeric and making tetrahydro curcumin generate in a biomass, After collecting the yeast which contains tetrahydro curcumin with techniques, such as centrifugal separation, tetrahydro curcumin content yeast foodstuffs can be manufactured by drying this. The extract which obtained yeast containing the tetrahydro curcumin manufactured by this invention by drying or condensing a hot water extract or after carrying out an alcohol extract, It can use as a new food yeast extract which gave the function of tetrahydro curcumin as compared with the yeast extract currently conventionally manufactured aimed at obtaining nourishment.

[0060]Cooking oil containing tetrahydro curcumin, For example, the biomass of the microorganism which has the activity which changes curcumin into tetrahydro curcumin under existence of O/W emulsion, If cooking oil is mixed and cooking oil is subsequently collected by techniques, such as centrifugal separation, after making culture medium or those treatment objects act on the constituent containing curcumin or a turmeric and making tetrahydro curcumin generate, Cooking oil containing tetrahydro curcumin can be manufactured. The cooking oil in which the solubility of the tetrahydro curcumin to cooking oil contains tetrahydro curcumin with a low content of the curcumin as \*\*\*\*\* by using this method since it is high as compared with curcumin is obtained. Since tetrahydro curcumin has an antioxidant action, it can use as extremely stable cooking oil in which air oxidation does not happen easily, and also cooking oil containing the obtained tetrahydro curcumin can be used as cooking oil provided with various physiological functions of tetrahydro curcumin.

[0061]The alcoholic beverage containing tetrahydro curcumin, For example, the biomass of the microorganism which has the activity which changes curcumin into tetrahydro curcumin under existence of O/W emulsion, After making culture medium or those treatment objects act on the constituent containing curcumin or a turmeric and making tetrahydro curcumin generate, When it is necessary to mix with beverage alcohol, to collect alcohol by techniques, such as centrifugal separation, subsequently, and to add sweetners, amino acid, sugars, a coloring agent, and flavors if needed and alcohol concentration needs to be raised further, it can manufacture by adding ethanol.

[0062]The wine or the Japanese sake containing tetrahydro curcumin, A grape or rice is fermented using wine yeast, sake yeast, etc. which have the activity which changes curcumin into tetrahydro curcumin, At the time in the middle of fermentation of the end of fermentation, for example under existence of O/W emulsion, after adding

curcumin or a turmeric and making tetrahydro curcumin generate, it can squeeze and manufacture. Although the anti-arteriosclerosis operation originating in the antioxidation activity of the red coloring matter contained in red wine or tannin, etc. attract attention, a color, the white wine which strengthened antioxidation activity, and Japanese sake can be manufactured by this invention, without affecting it clever. [0063]The fermentation dairy products containing tetrahydro curcumin, Milk etc. are fermented using the lactic acid bacteria which have the activity which changes curcumin into tetrahydro curcumin, and curcumin or a turmeric can be added and manufactured, for example under existence of O/W emulsion at the time in the middle of fermentation of the end of fermentation.

[0064]Any microorganisms can be used if it is a microorganism which has the activity which changes curcumin into tetrahydro curcumin as a microorganism used for this invention. As a microorganism, bacteria, a ray fungus, yeast, a filamentous bacterium, a mushroom, algae, etc. are used, for example.

[0065]As bacteria, for example An ASEROBAKUTA group (Acetobacter), Achromobacter (Achromobacter), an ASERO Bakter group (Arthrobacter), Bacillus (Bacillus), Bifidobacterium (Bifidobacterium), Brevibacterium (Brevibacterium), Cellulomonas (Cellulomonas), Chromobacterium (Chromobacterium), a SHITOBAKUTA group (Citobacter), Clostridium (Clostridium), Corynebacterium (Corynebacterium), Enterococcus (Enterococcus), the Ey Oui Near group (Erwinia), An ESSHIERISHIA group (Escherichia), Flavobacterium (Flavobacterium), Gluconobacter (Gluconobacter), Halobacterium (Halobacterium), Klebsiella (Klebsiella), a leuco NOSUTOKU group (Leuconostoc), the Micrococcus (Micrococcus), A PEJIOKOKKASU group (Pediococcus), Propionibacterium (Propionibacterium), A pro TAMINO Bakter group (Protaminobacter), the Providencia (Providencia), Pseudomonas (Pseudomonas), Serratia (Serratia), The SUTOREPUTO Bacterium (Streptobacterium), A streptococcus group (Streptococcus), Xanthomonas (Xanthomonas), A JIMOMONASU group (Zymomonas), the Lactobacillus group (Lactobacillus), a PEJIOKOKKASU group (Pediococcus), a Staphylococcus group (Staphylococcus), etc. are raised.

[0066]As a ray fungus, Actinoplanes (Actinoplanes), Micromonospora (Micromonospora), Streptomyces (Streptomyces), etc. are raised, for example. As yeast, for example A DEBARYOMAISESU group (Debaryomyces), Saccharomyces (Saccharomyces), Pichia (Pichia k),The Kluyveromyces group (Kluyveromyces), the genus Torulaspora (Torulaspora), The Candida group (Candida), a SHUBIA group (Ashbya), a BURETTANOMAISESU group (Brettanomyces), A Cryptococcus

(Cryptococcus), an EREMOTERIUMU group (Eremothecium), An ISSACHIENKIA group (Issatchenkia), a KUROKKERA group (Klockera), A RIPOMAISESU group (Lipomyces), a METOSHUNIKOUIA group (Metschnikowia), A load TORUA group (Rhodotorula), the Schizosaccharomyces group (Shizosaccharomyces), gigot Saccharomyces (Zygosaccharomyces), etc. are fried.

[0067]As a filamentous bacterium, for example The Acremonium group (Acremonium), An Actinomucor group (Actinomucor), an Aspergillus (Aspergillus),The Aureobasidium group (Aureobasidium), the Baku Serra group (Backusella), Genus botrytis (Botrytis), a CHARARA group (Chalara), A KURABISEPUSU group (Claviceps), a col CHISHIUMU group (Corticium), KURIFONEKU --- doria --- a group (Cryphonectria) and the Eurotium group (Eurotium). A fusarium group (Fusarium), a GEOTORICHUMU group (Geotrichum), A monas dregs group (Monascus), a mol CHIERERA group (Mortierella),A Mucor (Mucor), a MIROTESHIUMU group (Myrothecium), The New Ross Poral group (Neurospora), a PAESHIROMAISESU group (Paecilomyces), A Penicillium (Penicillium), a PESUTAROCHIOPUSU group (Pestalotiopsis), a RIZOMU call group (Rhizomucor), Rhizopus (Rhizopus), the Sclerotinia group (Sclerotinia), A synthesizer FARASUTORUMU group (Syncephalastrum), Trichoderma (Trichoderma), etc. raise, and there are.

[0068]As a mushroom, for example An agaricus group (Agaricus), an AGUROSHIBE group (Agrocybe), An aluminum rallier group (Armillaria), an auricularia group (Auricularia), The Fulham Lina group (Flammulina), the Ganoderma group (Ganoderma), A GURIFORA group (Grifola), a HIPUSHIJIGASU group (Hypsizigus), An yl PEKKUSU group (Irpex), a wrench NURA group (Lentinula), A REPISUTA group (Lepista), a RIOFIRIUMU group (Lyophyllum), A MAIKOREPUTODONOIDESU group (Mycoleptodonoides), The Naematoloma group (Naematoloma), a PANERUSU group (Panellus), the Pori Ota group (Pholiota), a PURYUOTSUSU group (Pleurotus), the Pycnoporus group (Pycnoporus), a TOREMERA group (Tremella), and tricot --- a ROMA group (Tricholoma), the Bolu Bali Ella group (Volvariella), etc. are raised.

[0069]As algae, for example An ANARIPUSU group (Anaripus), a KONDORASU group (Chondrus), A ray SENIA group (Eisenia), a you SHUMA group (Eucheuma), A Furcellaria group (Furcellaria), a JIGACHINA group (Gigartina), A HIJIKIA group (Hizikia), a KUJIERA manners group (Kjellmaniella), The Laminaria group (Laminaria), a macro KURISUCHISU group (Macrocrystis), A PETARONIA group (Petalonia), a PORUFIRA group (Porphyra), a ROJIMENISU group (Rhodymenis), a SHITOSHI phone group (Scytosiphon), Spirulina (Spirulina), a UNDARIA group (Undaria), etc. are raised.

[0070]Specifically, microorganisms, such as staphylococci, such as lactic acid

bacteria, such as yeast, such as a DEBARYOMAISESU group, *Saccharomyces*, *Pichia*, the *Kluyveromyces* group, the genus *Torulaspora*, and the *Candida* group, the *Lactobacillus* group, and a PEJIOKOKKASU group, and a *Staphylococcus* group, are used. Still more concretely as a suitable strain, For example, DEBARYOMAISESU alder ZENII. (*Debaryomyces*) *hansenii* ATCC-20261, DEBARYOMAISESU alder ZENII (*Debaryomyces hansenii*) IFO-0094, the product made by *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) dried yeast Lallmand, *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) IAM-4500 and *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) IAM-4519, *Saccharomyces SEREBISHIAE*. (*Saccharomyces cerevisiae*). ATCC-7754, *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) FERM-P-6189, and *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) IFO-2044, *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) ATCC-20018, *Saccharomyces SEREBISHIAE*. (*Saccharomyces cerevisiae*). FERM-P-6213, *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*) FERM-P-6214, and *Saccharomyces SEREBISHIAE* (*Saccharomyces cerevisiae*). FERM-P-7614, *Saccharomyces SEREBISHIAE*. (*Saccharomyces cerevisiae*). FERM-P-7615, *Pichia subperi* KYUROSU (*Pichia subpelliculosa*) ATCC-16766, *Pichia ANOMARA* (*Pichia anomala*) ATCC-2149, and the *Pichia MEMBURANAE faciae* --- nth (*Pichia*) *membranaefaciens* IAM-4986, *Pichia MEMBURANAE faciae* nth (*Pichia membranaefaciens*) ATCC-36908, *Pichia KURUIBERI* (*Pichia kluyveri*) ATCC-9768, *Kluyveromyces MAKUSHI* anus (*Kluyveromyces marxianus*) IFO-0433, The *Kluyveromyces MAKUSHI* anus. (*Kluyveromyces marxianus*) IFO-1090, *Kluyveromyces police* PORUSU. (*Kluyveromyces polysporus*) ATCC-22028, *Torulaspora Dell Vrutkey* (*Torulaspora delbrueckii*) IAM-4816, and *Candida UCHIRISU* (*Candida utilis*) ATCC-9950, *Candida FAMATA* (*Candida famata*) ATCC-2560, *Lactobacillus plantarum* (*Lactobacillus plantarum*) (made by a harmony high foods company) LPT. *Staphylococcus cull* NOSASU (*Staphylococcus carnosus*) M72 (made by TEXEL), PEJIOKOKKASU reed JIRAKUCHISHI (*Pediococcus acidilactici*) P2M120 (made by TEXEL) and *Staphylococcus KISHIRO SASU* (*Staphylococcus xylosum*2) P2M120 (made by TEXEL) are raised.

[0071] These of any strain are independent, or can be mixed and used. If it is a strain in which the variant to which these strains were mutated in the artificial variation method, for example, UV irradiation, X-ray irradiation, variation induction agent processing, gene manipulation, etc., or the variant which varied automatically also has the capability to transform curcumin to tetrahydro curcumin, it can use for this

invention.

[0072]If it is a culture medium which can grow these microorganisms by the culture medium used for culture of usual yeast, bacteria, etc. for culture of these microorganisms, any culture media, such as a natural medium, a semisynthetic medium, a synthetic medium, etc. containing a carbon source, a nitrogen source, and other nutrients, can be used. as a carbon source, starch, dextrin, sucrose, glucose, mannose, fructose, raffinose, rhamnose, inositol, lactose, xylose, arabinose, mannitol, molasses, etc. are raised -- these -- it can be independent, or it can combine and can use. Hydrocarbon, alcohols, organic acid, etc. may be used depending on the utilization ability of a bacillus.

[0073]as a nitrogen source, ammonium chloride, ammonium nitrate, ammonium sulfate, sodium nitrate, urea, peptone, a meat extract, a yeast extract, dried yeast, corn steep liquor, soybean flour, casamino acids, etc. are raised --- these -- it can be independent, or it can combine and can use. as mineral, sodium chloride, potassium chloride, magnesium sulfate, calcium carbonate, potassium dihydrogen phosphate, ferrous sulfate, a calcium chloride, manganese sulfate, sulfate of zinc, copper sulfate, etc. are raised -- these -- it can be independent, or it can combine and can use.

[0074]as a minor constituent, amino acid, such as vitamins, such as biotin, thiamin, or nicotinic acid, beta-alanine or glutamic acid, is raised -- these -- it can be independent, or it can combine and can use. As cultivation, the liquid culture method, especially the depths spin-culturing method are suitable. culture -- the temperature of 10-80 \*\* -- desirable -- 10-60 \*\* -- especially -- desirable -- 20-40 \*\*, pH 2-11, and pH 3-10 -- it is preferably carried out by pH 5-8, and usually carries out for one to seven days. An ammonia solution, an ammonium carbonate solution, etc. are used for the pH adjustment of a culture medium.

[0075]The concentrate of the culture medium of the microorganism which has the activity which changes curcumin into tetrahydro curcumin as a treatment object of the culture medium used for this invention, a dry matter, a frozen matter, a refrigeration thing, a freeze-drying thing, a heating substance, pressurized material, ultrasonic debris, a surface-active agent or an organic solvent treatment object, a lytic enzyme treatment object, etc. are raised. The dry matter of the biomass of the microorganism which has the activity which changes curcumin into tetrahydro curcumin as a biomass treatment object, The enzyme refined from a frozen matter, a refrigeration thing, a freeze-drying thing, a heating substance, pressurized material, ultrasonic debris, a surface-active agent or the organic solvent treatment object, the lytic enzyme treatment object, the immobilized cell, or the biomass etc. are raised.

[0076]A proteinic general purification method can be used for refining of the enzyme from a biomass. For example, crushing of the biomass by homogenizer, a glass bead, the ammonia dissolution, enzymatic process, etc., Recovery of the enzyme by recovery of the enzyme liquid by filtration, centrifugal separation, etc., curing salting, organic solvent precipitate, an antibody, etc., it is independent about the chromatography using separation by concentration by dialysis etc., ultrafiltration, the gel filtration, the electrophoresis method, and the liquid phase distributing method, an ionic exchanger, adsorbent, affinity adsorbent, etc., a batch method, crystallization, etc. -- it is -- it can combine and use.

[0077]Curcumin can be obtained from for example, sigma Aldrich Japan as a reagent. although not limited especially as a constituent containing curcumin -- curcmae rhizoma -- the constituent produced by processing the vegetation belonging to a group is raised. curcmae rhizoma -- as vegetation belonging to a group, vegetation, such as curcmae aromaticae rhizoma (commonly called curcmae rhizoma in spring.), curcmae rhizoma (commonly called curcmae rhizoma in autumn.), and a zedoary, is fried, for example. Although a disposal method in particular is not limited, methods, such as grinding, desiccation, and extraction, are raised, for example. As a constituent containing curcumin, general food, such as curry powder and turmeric tea, is also raised, for example.

[0078]The constituent produced by adding the constituent containing curcumin or curcumin is also raised to the foodstuffs, the alcoholic beverage, feed, and cosmetics which do not contain curcumin as a constituent containing curcumin. As the foodstuffs, the alcoholic beverage, feed, and cosmetics which do not contain curcumin, beverage alcohol, cooking oil, fruit juice, etc. are raised. The constituent containing curcumin and curcumin can obtain a commercial item easily as foodstuffs.

[0079]Although working example explains this invention below, this example does not restrict this invention.

[0080]

[Example]63.6 g of preparation glycerin (made by Kishida Chemical Co., Ltd.) of the conversion reaction 1 O/W emulsion under working example 1 O/W-emulsion coexistence, 5 g of polyglycerin mono- olate (the poem J-0381, the Riken Vitamin Co., Ltd. make), It mixed within the cup for homogenizers and 1 g of enzymatically decomposed lecithin (El Myser AC, TSURU lecithin industrial incorporated company make) and the pure water 20g were stirred almost uniformly with the homogenizer (AM-7 type, the product made by NISSEI). 10g of MCT(s) (PANASETO 810, Nippon Oil & Fats Co., Ltd. make) were added to this, it stirred for 10 minutes at 17,000 rpm, and

the O/W emulsion which does not contain curcumin was prepared. The mean particle diameter of this O/W emulsion was 110 nm (particle-diameter measurement: model N4MD, product made by COULTER).

[0081]2) Conversion reaction Debaryomyces Hansenii (PRISCA and LACTO LABO) by the biomass under O/W emulsion coexistence by YM culture medium (made by NISSUI). Two day and night were cultivated at 25 °C (a 0.03g biomass / ml), within a 2.0-ml Eppendorf tube, it centrifuged for 10 minutes at 12,000 rpm, and the biomass was obtained. It was suspended by the O/W emulsion which does not contain the above-mentioned curcumin so that this biomass may be set to a 0.4g biomass / ml, and could be 1 ml. 2 mg of curcumin powder (made in sigma Aldrich Japan) was added to this suspension, and it was kept warm, shaking violently at 200 rpm for 48 hours using a shaking culture machine (TC-C-500 R form, the Takasaki science instrument company make) at 37 °C.

[0082]The tetrahydro curcumin in reaction mixture was analyzed in the following HPLC measuring condition.

HPLC condition: --- solvent: --- 50% of 0.05% trifluoroacetic acid content acetonitrile solution rate-of-flow: --- 0.66ml/min tetrahydro curcumin generated detection:280nm, as a result 48 hours afterward by 1-ml/.

[0083]adding the curcumin (made in sigma Aldrich Japan) of 2 g of preparation of the oil containing working example 21 curcumin to 10-g MCT (PANASETO 810, Nippon Oil & Fats Co., Ltd. make) --- an oil bath (BO-11 type.) In the product made by Yamato, it heated for 10 minutes and 115 °C of curcumin content oils were prepared.

[0084]2) The preparation glycerin 63.6g of curcumin content O/W emulsion (G), 5 g of polyglycerin mono- olate (the poem J-0381, the Riken Vitamin Co., Ltd. make), 1 g enzymatically decomposed lecithin (El Myser AC, TSURU lecithin industrial incorporated company make) and water It mixed within the cup for homogenizers and 20 g was stirred almost uniformly with the homogenizer (AM-7 type, the product made by NISSEI). This curcumin content oil was stirred for 10 minutes by 17,000 rpm of whole-quantity \*\*\*\*\* to this, and O/W emulsion-ized curcumin was manufactured. The curcumin concentration in this O/W emulsion was 2.5mg/ml, and was 150 nm (particle-diameter measurement: model N4MD, product made by COULTER) in mean particle diameter. This O/W emulsion is only called O/W emulsion (G) below.

[0085]3) 5 g of preparation polyglycerin mono- olate (the poem J-0381, the Riken Vitamin Co., Ltd. make) of curcumin content O/W emulsion (W), 1 g of enzymatically decomposed lecithin (El Myser AC, TSURU lecithin industrial incorporated company make), water It mixed within the cup for homogenizers and 83.6 g was stirred almost



uniformly with the homogenizer (AM-7 type, the product made by NISSEI). This curcumin content oil was stirred for 10 minutes by whole-quantity \*\*\*\*\* 17,000 rotation to this, and O/W emulsion-ized curcumin was manufactured. The curcumin concentration in this O/W emulsion was 2.5mg/ml, and was 150 nm (particle-diameter measurement: model N4MD, product made by COULTER) in mean particle diameter. Hereafter, this O/W emulsion is only called O/W emulsion (W).

[0086]4) Conversion reaction Debaryomyceshansenii (PRISCA, product made by LACTO LABO) by the biomass under O/W emulsion coexistence by YM culture medium (made by NISSUI). Two day and night were cultivated at 25 \*\* (a 0.03g biomass / ml), within a 2.0-ml Eppendorf tube, it centrifuged for 10 minutes at 12,000 rpm, and the biomass was obtained. This biomass to 200microl of an O/W emulsion-ized curcumin undiluted solution (100%) or an O/W emulsion-ized curcumin water diluent (25 and 50%). It was suspended so that it might be set to a 0.4g biomass / ml, it was considered as reaction mixture, and it was kept warm, shaking this violently at 200 rpm for 4 hours using a shaking culture machine (TC-C-500 R form, the Takasaki science instrument company make) at 30 \*\*. 4 hours afterward, 800microl addition of the ethanol was carried out, the vortex was carried out, HPLC analysis of the supernatant liquid obtained by centrifuging for 1 minute at 12000 rpm was conducted by the following condition, and the curcumin in reaction mixture and tetrahydro curcumin concentration were computed.

[0087]The tetrahydro curcumin in reaction mixture was analyzed in the above-mentioned HPLC analysis condition. As a result, when O/W emulsion was added by capacity 25%, 50%, and 100%, 0.047mg/ml - 0.275mg/ml tetrahydro curcumin generated. A result is shown in the 1st table.

[0088]Comparative example 1Debaryomyceshansenii (PRISCA, product made by LACTO LABO) by YM culture medium (made by NISSUI). Two day and night were cultivated at 25 \*\* (a 0.03g biomass / ml), within a 2.0-ml Eppendorf tube, it centrifuged for 10 minutes at 12,000 rpm, and the biomass was obtained. To water 200mul, are suspended so that it may be set to a 0.4g biomass / ml, and this biomass is made into reaction mixture at it, 1 / 20 capacity addition of the 4mg/ml of curcumin ethanol solution were carried out at this, and it was kept warm, shaking this violently at 200 rpm for 4 hours using a shaking culture machine (TC-C-500 R form, the Takasaki science instrument company make) at 30 \*\*. The ethanol concentration in reaction mixture is 5%. 4 hours afterward, 800microl addition of the ethanol was carried out, the vortex was carried out, HPLC analysis of the supernatant liquid obtained by centrifuging for 1 minute at 12,000 rpm was conducted by the following

condition, and the curcumin in reaction mixture and tetrahydro curcumin concentration were computed. The HPLC analysis condition is the same as working example 1. A result is shown in the 1st table.

[0089]

[Table 1]

第 1 表

			初期U1濃度 (mg/ml)	生成THU1濃度 (mg/ml)
実施例2	エマルジ ョン濃度	25	0.5	0.086
		50	1.0	0.168
		100	2.0	0.275
比較例1			0.2	0.051

[0090] THU1 can be made to generate efficiently if O/W emulsion-ized curcumin is used.

[0091] The microorganism conversion reaction biomass method of preparation using working example 3 O/W-emulsion-ized curcumin and concentration were performed like working example 1. Ten things suspended with the O/W emulsion-ized curcumin undiluted solution of 200microl in this biomass were prepared (cell mass concentration set to a 0.4g biomass / ml), and it was kept warm, shaking violently at 30 \*\*. It added two ethanol 800microl at a time 4, 8, and 24 or 72 hours afterward, the reaction was suspended, and HPLC analysis was presented with the supernatant liquid obtained by centrifuging for 1 minute at 12,000 rpm.

[0092] In the reaction with initial U1 concentration [ using O/W emulsion (G) ] of 2.5mg/ml, THU1 generated amount increased till 72 hours, and the value became in ml and 2.3mg /, 10 or more times of what added the ethanol solution. A result is shown in the 2nd table.

[0093] Comparative example 2 biomass was suspended with water (cell mass concentration set to a 0.4g biomass / ml), and it was kept warm, shaking violently what carried out 1 / 10 capacity addition of the 18mg/ml of curcuminethanol suspension (curcumin final concentration of 1.8mg/ml) at 30 \*\*. It added two ethanol 800microl at a time 4, 8, and 24 or 72 hours afterward, the reaction was suspended, and HPLC analysis was presented with the supernatant liquid obtained by centrifuging for 1 minute at 12,000 rpm. A result is shown in the 2nd table.

[0094]

[Table 2]

第 2 表

反応時間 (時間)	実施例 3		比較例 2	
	U1	THU1	U1	THU1
0	2.54	0	1.77	0
4	2.31	0.53	1.63	0.1
8	1.92	1.24	1.63	0.15
24	1.06	1.55	1.4	0.16
72	0.52	2.3	1.4	0.13

[0095]In the conventional ethanol method, almost all U1 was not changed but it remained as shown in the 2nd table. As shown in the 2nd table, in the conversion reaction using O/W emulsion-ized curcumin. Not less than 90% of the curcumin added 72 hours after the reaction has been changed into tetrahydro curcumin to having changed into tetrahydro curcumin by the conversion reaction using the curcumin dissolved in ethanol 10% or less of the curcumin added 72 hours after the reaction.

[0096]According to the application experimental-medicine separate volume bio-manual series 10 to the enzyme reaction of working example 4

O/W-emulsion-ized curcumin "gene testing method by yeast" (Masayuki Yamamoto edit, Yodosha), PRISCA biomass crushing liquid (crude enzyme liquid) was created. That is, 25 ml (a 0.045g biomass / ml) of culture medium which cultivated PRISCA by YM culture medium is centrifuged (6,000 rpm). It was suspended by DIW2ml which ice-cooled the precipitate produced by carrying out for 20 minutes, and after moving and carrying out the vortex to the Eppendorf tube and washing to it, centrifugation was carried out again and biomass 1.1g/ml was obtained. 1.2-ml Lysis buffer (the following presentation) and 1.6 ml of glass beads were added to this biomass, and it cooled in Hikami. This was violently stirred with the vortex mixer (MULTI BEADS SHOCKER MB-200 and Yasui Kikai Corp.). After stirring for 30 seconds, the operation cooled in Hikami during 1 minute was repeated 10 times. Then, the supernatant liquid centrifuged and (for 15,000 rpm and 30 minutes, 4 \*\*) obtained was collected, and this was made into crude enzyme liquid.

[0097]Lysis buffer presentation (concentration is final concentration) : 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 5mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 5% glycerol, and 3 mmol/L Dithiothreitol, 1 mmol/L PMSF (autoclave of 120 \*\* of the above is carried out for 20 minutes, and it is saved.) Dithiothreitol and PMS are added just before use.

[0098]To this crude enzyme liquid 100mul, 0.5 mol/L ATP, 100 mmol/L NADH, 1 / 10 capacity addition of the O/W emulsion-ized curcumin (G) or O/W emulsion-ized curcumin (W) which manufactured 100 mmol/L NADPH in 1/100 capacity and working

example 2 respectively were carried out, and shaking incubation was carried out at 37 \*\*. It diluted with the ethanol of capacity 10 times 0.5, 1, and 2 or 24 hours afterward, and HPLC was presented with centrifugal supernatant liquid. A result is shown in the 3rd table.

[0099]

[Table 3]

第 3 表

反応時間 (時間)	クルクミン (G)		クルクミン (W)	
	U1	THU1	U1	THU1
0	0.18	0.00	0.23	0.0
0.5	0.08	0.09	0.13	0.12
1	0.04	0.17	0.05	0.19
2	0.01	0.19	0.03	0.22
24	0.01	0.18	0.03	0.20

[0100]By using O/W emulsion-ized curcumin, curcumin was changed into tetrahydro curcumin nearly thoroughly 24 hours afterward.

[0101]Comparative example 3 *Debaryomyces Hansenii* (PRISCA and LACTO LABO) by YM culture medium (made by NISSUI). Two day and night were cultivated at 25 \*\* (0.03g/(ml)), within a 2.0-ml Eppendorf tube, it centrifuged for 10 minutes at 12,000 rpm, and the biomass was obtained. This biomass so that it may be suspended so that it may become [ ml ] in 0.4g /, and it may be considered as reaction mixture and the concentration in the reaction mixture of curcumin may become [ ml ] this in 0.1mg /by water 200mul, 2mg/ml of curcumin ethanol solution 1/20 capacity (5% of ethanol final concentration), 1mg/ml of curcumin ethanol solution Or 1/10 capacity (10% of ethanol final concentration), Or 1 / 5 capacity addition of the 0.5mg/ml of curcumin ethanol solution were carried out (20% of ethanol final concentration), and it was kept warm, shaking this violently at 200 rpm for 4 hours using a shaking culture machine (the Takasaki science instrument, TC-C-500 R form) at 30 \*\*. 4 hours afterward, 800microl addition of the ethanol was carried out, the vortex was carried out, HPLC analysis of the supernatant liquid obtained by centrifuging for 1 minute at 12,000 rpm was conducted by the following condition, and the curcumin in reaction mixture and tetrahydro curcumin concentration (THU1 concentration) were computed. The HPLC analysis condition is the same as working example 1. A result is shown in the 4th table.

[0102]

[Table 4]

第 4 表

エタノール濃度(%)	THU1濃度( $\mu$ g/ml)
5	70
10	70
20	15

[0103]When ethanol concentration was 5% and 10%, to the generated amount of THU1 having been 70 microg/ml, at 20%, the generated amount of THU1 was low in ml and 15 microg /, and a possibility that the enzyme of the system of reaction was deactivated in high-concentration ethanol was suggested.

[0104]Comparative example 4 *Debaryomyces Hansenii* (PRISCA and LACTO LABO) by YM culture medium (made by NISSUI). Two day and night were cultivated at 25 \*\* (a 0.03g biomass / ml), within a 2.0-ml Eppendorf tube, it centrifuged for 10 minutes at 12,000 rpm, and the biomass was obtained. By water 20mul, are suspended so that it may be set to a 0.4g biomass / ml, and this biomass is made into reaction mixture, To this, 2mg [ // ml ], 4mg [ // ml ] or 6mg [ // ml ], 8mg [ // ml ], or 10mg/ml of curcumin ethanol solution so that the ethanol concentration in reaction mixture may be 5%, 1 / 20 capacity addition was carried out, and it was kept warm, shaking this violently at 200 rpm for 4 hours using a shaking culture machine (TC-C-500 R form, the Takasaki science instrument company make) at 30 \*\*, 4 hours afterward, 800microl addition of ethanol was done, it shook in the vortex, HPLC analysis of the supernatant liquid obtained by centrifuging for 1 minute at 12,000 rpm was conducted by the following condition, and the curcumin in reaction mixture and tetrahydro curcumin concentration were computed. The HPLC analysis condition is the same as working example 1. A result is shown in the 5th table.

[0105]

[Table 5]

第 5 表

添加クルクミン濃度 (mg/ml)	反応液中濃度 ( $\mu$ g/ml)	生成THU1 ( $\mu$ g/ml)
2	100	75
4	200	120
6	300	120
8	400	90
10	500	90

[0106]The curcumin which melted and remained was observed in what added the curcumin ethanol solution of high concentration [ 4mg/ml ]. THU1 concentration generated even if generation tetrahydro curcumin (THU1) concentration shows the

120 microg/ml maximum at the time of U1 [ 4mg / ml / and 6mg/ml ] and raises addition curcumin concentration did not increase.

[0107]

[Effect of the Invention]According to this invention, it cannot be based on the technique of chemosynthesis hydrogenation using a catalyst, but the manufacturing method of the constituent containing the manufacturing method of industrial tetrahydro curcumin using a microbial cell, cultures, or those treatment objects and tetrahydro curcumin can be provided.

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[Translation done.]

(19)



JAPANESE PATENT OFFICE

## PATENT ABSTRACTS OF JAPAN

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**NOMOTO FUMIKI**

**(54) TRANSESTERIFICATION****(57) Abstract:**

**PROBLEM TO BE SOLVED:** To quantitatively carry out a transesterification reaction useful for producing a fatty acid ester etc., without using a solvent by transesterifying an ester with an alcohol by using an esterase in an aqueous system.

**SOLUTION:** An ester (e.g. oils and fats, etc.), is transesterified with an alcohol in an aqueous system containing 1-20 wt.% of water in the reaction system by using an esterase (e.g. lipase) so as to carry out the transesterification between the ester

and the alcohol by a method for producing an automobile fuel (biodiesel fuel) from naturally occurring oils and fats of animal and plant and microorganism, especially waste oil instead of fossil fuel from the viewpoint of environment problem. Conventionally in a transesterification reaction, the reaction is carried out by eliminating water as much as possible but it is found that a transesterification reaction is performed even in a system sufficiently containing water and yet approximately quantitatively.

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CLAIMS

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[Claim(s)]

[Claim 1]An ester interchange method performing an ester exchange reaction between ester and alcohol in a hydrous system using esterase.

[Claim 2]A way according to claim 1 said hydrous system is a system which contains 1 to 20% of the weight of water in the system of reaction.

[Claim 3]A method according to claim 1 or 2 by which said reaction is performed by a non-solvent system.

[Claim 4]A method given in a paragraph of one of Claims 1-3 said whose esterase is lipase and in which said ester is fats and oils.

[Claim 5]A manufacturing method of fatty acid ester including a process to which fats and oils and alcohol are made to react under existence with water and lipase.

[Claim 6]A method according to claim 5 by which said water is contained in the system of reaction one to 20% of the weight.

[Claim 7]A method according to claim 5 or 6 by which said reaction is performed by a non-solvent system.

[Claim 8]A method given in a paragraph of one of Claims 5-7 in which said fats and oils are waste oil.

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[Translation done.]



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DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[Field of the Invention]This invention relates to the ester interchange method by esterase. This invention relates to the method of performing an ester interchange by a hydrous system, in more detail.

[0002]

[Description of the Prior Art]In recent years, the trial which manufactures motor fuel (what is called biodiesel fuel) is made from the fats and oils of the animals and plants which replace with a fossil fuel and exist naturally from a viewpoint of an environmental problem, and a microorganism. Since especially waste oil will cause an environmental problem if it is thrown away, it is thought that manufacture of the biodiesel fuel from waste oil contributes to recycling of resources and solution of an environmental problem greatly.

[0003]There are the chemical method and a biological method in manufacture of the biodiesel fuel from such natural fats and oils (waste oil). The chemical method is a hot energy many consumption type reaction, and there is a problem of processes, like a neutralization process is needed. Then, the energy few consumption type biological method is examined by ordinary temperature.

[0004]A biological method is a method of dissolving in a solvent (for example, hexane) and making fats and oils reacting to lipase under existence of alcohol chiefly. The enzyme in which lipase hydrolyzes fats and oils into fatty acid and triglyceride under existence of water uses a solvent.

It is because it is thought that a hydrolysis reaction advances by existence of water

and an ester exchange reaction does not advance easily on the occasion of the ester rearrangement (ester interchange) reaction of fats and oils and alcohol.

Therefore, in the ester rearrangement reaction (ester exchange reaction), it is thought that the system in which water does not exist, or a minute amount moisture system is indispensable, and the method of using a solvent is recommended. Therefore, also in a biological method, there are recovery of a solvent and a problem on processes, such as prevention from explosion, on a process.

[0005]In order to solve this problem, examination by the system which does not use a solvent is made. For example, although fatty acid ester is obtained using branching alcohol, each of these is expensive alcohol and the example which showed high conversion in industrial alcohol, such as cheap methanol and ethanol, is not reported to 73 JAOCS(s) and 1191-1195 pages (1996).

[0006]Since it is thought on the other hand that an esterolysis reaction advances with the moisture contained in fats and oils or waste oil when using the fats and oils and waste oil of animals and plants and a microorganism, removal of moisture poses a problem.

[0007]Thus, in order to perform efficiently fats and oils or biodiesel fuel from waste oil, it is necessary to perform an ester exchange reaction by a non-solvent system using cheap alcohol, such as methanol, but, taking the influence of moisture into consideration, and, All of the problem which uses cheap alcohol according to the problem and the non-solvent system of the influence of water under the present circumstances are unsolved.

[0008]

[Problem(s) to be Solved by the Invention]Then, the method of performing an ester exchange reaction by a non-solvent system using cheap alcohol, such as methanol, is called for, taking the influence of moisture into consideration, in order to perform efficiently fats and oils or biodiesel fuel from waste oil.

[0009]

[Means for Solving the Problem]As a result of inquiring that the above-mentioned problem should be solved, this invention persons, Under existence of water, the conventional established theory that an ester exchange reaction hardly advanced was reversed, and an ester exchange reaction's advancing also under existence of water and an ester exchange reaction found out that it could carry out under a non-solvent using cheap alcohol, and completed this invention. An ester exchange reaction in a hydrous system which was not considered conventionally is performed by this invention, and composition of fatty acid ester (biodiesel fuel) from fats and oils

containing moisture of animals and plants and microorganism fats and oils, or waste oil is performed efficiently.

[0010]That is, this invention relates to an ester interchange method performing an ester exchange reaction between ester and alcohol in a hydrous system using esterase.

[0011]In a desirable embodiment, said hydrous system is a system which contains 1 to 20% of the weight of water in the system of reaction.

[0012]In a desirable embodiment, a reaction is performed by a non-solvent system.

[0013]In a desirable embodiment, said esterase is lipase and said ester is fatty acid ester.

[0014]This invention relates to a manufacturing method of fatty acid ester which includes again a process to which fats and oils and alcohol are made to react under existence with water and lipase.

[0015]In a desirable embodiment, said water is contained in the system of reaction one to 20% of the weight.

[0016]In a desirable embodiment, said reaction is performed by a non-solvent system.

[0017]In a desirable embodiment, fats and oils are waste oil.

[0018]

[Embodiment of the Invention]Esterase, alcohol, water, and ester are contained in the system of reaction used by this invention.

[0019]In this invention, it is the greatest feature that water is in the system of reaction. This invention is epoch-making if the point in which were trying not to make water exist as much as possible is taken into consideration, since hydrolysis of ester will advance, if water exists conventionally. Water is contained 0.3% of the weight or more in the system of reaction, and is contained five to 8% of the weight one to 10% of the weight one to 20% of the weight preferably. When calling it a hydrous system, the case where it contains 0.3% of the weight or more in the system of reaction is said.

[0020]Typically, the ester interchange method of this invention mixes these fats and oils and alcohol, adds enzyme solution to this, or performs it by the suitable method of adding moisture to the reaction mixture of fats and oils, alcohol, and the enzyme of dryness. Fats and oils and an enzyme form an emulsion and a reaction is considered to go on by the interface. Therefore, it is thought that advance of a reaction is controlled by the size of an emulsion, enzyme concentration, etc. The moisture content contained in the above-mentioned system of reaction should just also determine the optimal moisture content in consideration of such a point. Therefore, it may be contained exceeding 20 % of the weight made preferred [ water ].

[0021]Solvents (for example, hexane etc.) may be contained in the above-mentioned system of reaction. Although the case where a solvent is not contained is called non-solvent system, a non-solvent system is a meaning which does not contain the solvent for dissolving fats and oils, and the alcohol used for an ester exchange reaction is not a solvent told to this invention.

[0022]In this invention, esterase says the enzyme which hydrolyzes ester and contains carboxylesterase, peptidase, etc. The typical example of carboxylesterase is lipase. Lipase acts on glyceride and the enzyme which has the capability to decompose into glycerin or partial glycerides, and fatty acid is said.

[0023]Although lipase is taken for an example and this invention is explained hereafter, it cannot be overemphasized that it is applicable to other esterase.

[0024]The origin of lipase is not asked. Regardless of the shape of an enzyme, powder may be sufficient and it may be fixed. It contains, also when using the microorganism which produces lipase, and the immobilized microorganism which fixed the microorganism as an enzyme agent as it is. In these, it is the most desirable from a field, like a reuse is made with prompt mass transfer [ in / in the fixed lipase / reaction time ].

[0025]Lipase is 1 and 3. - It may be specific or may be nonspecific. From the field of manufacture of fatty acid ester, the more nearly nonspecific one is preferred. As lipase, for example A RIZOMU call (Rhizomucor) group, The Mucor (Mucor) group, an Aspergillus (Aspergillus) group, The filamentous bacterium belonging to the Rhizopus (Rhizopus) group, a penicillium (Penicillium) group, etc., Lipase originating in animals belonging to yeast belonging to the Candida (Candida) group, the Pichia (Pichia) group, etc., the Pseudomonas (Pseudomonas) group, a Serratia (Seratia) group, etc., such as bacteria and the pig pancreas, is mentioned.

[0026]Commercial lipase is also used. Hereafter, although illustrated, it is not limited to these. As lipase of filamentous-bacterium origin, trade name RIRIPAZE A-10FG (Rhizopus japonicus origin: Seikagaku, Inc., Nagase Brothers), The trade name lipase F (Rhizopus ORIZE origin: Amano Pharmaceuticals), the trade name lipase M (Mucor Java NIKASU origin: Amano Pharmaceuticals), etc. are mentioned.

[0027]As lipase of bacteria origin, a trade name SM enzyme (Serratia marcescens origin: Seikagaku, Inc., Nagase Brothers), the trade name lipase AH (Pseudomonas cepacia origin: Amano Pharmaceuticals), trade name lipase P: (Seikagaku, Inc., Nagase Brothers), etc. are mentioned.

[0028]As lipase of yeast origin, the trade name lipase L (Candida-lipolytica origin: Amano Pharmaceuticals), the lipase OF (Candida rugosa origin: the Meito Sangyo Co.,

Ltd.), etc. are mentioned.

[0029]The method of fixing these lipase in a carrier is publicly known. As a carrier, ion-exchange resin, a ceramic carrier, a glass bead, activated carbon, etc. are mentioned. When endurance, compatibility with lipase, etc. are taken into consideration, ion-exchange resin, a ceramic carrier, etc. are the most preferred. As a fixing method, although an entrapping elasticity, cross-linking method, physical adsorption process, and ion adsorption process, a hydrophobic bond method, etc. are mentioned, a cross-linking method and a hydrophobic bond method are the most preferred.

[0030]The commercial fixed lipase 435, for example, Novozym, and Lipozyme IM60 (all are the Novo Nordisk make) are used suitably for this invention.

[0031]As a microorganism, a filamentous bacterium, bacteria, yeast, etc. which produce lipase are mentioned. As a filamentous bacterium, an *Aspergillus* (*Aspergillus*) group, a galacto married-woman (*Galactomyces*) group, The microorganism belonging to a *Geotrichum* (*Geotricum*) group, the *Mucor* (*Mucor*) group, the *Phycomyces* (*Phycomyces*) group, a RIZOMU call (*Rhizomucor*) group, a penicillium (*Penicillium*) group, the *Rhizopus* (*Rhizopus*) group, etc. is mentioned.

[0032]As bacteria, the bacteria belonging to the *Pseudomonas* (*Pseudomonas*) group, the *Alcaligenes* (*Alkaligenes*) group, etc. are mentioned.

[0033]As yeast, yeast belonging to the *Candida* (*Candida*) group, a *Cryptococcus* (*Cryptococcus*) group, the *Pichia* (*Pichia*) group, the *Rhodotorula* (*Rhodotorula*) group, and the *Yarrowia* (*Yarrowia*) group is mentioned.

[0034]These microorganisms are illustration and it cannot be overemphasized that the microorganism of this invention is not limited to the microorganism of these illustration.

[0035]The above-mentioned microorganism may be fixed and used. Immobilization is performed by the method which a person skilled in the art uses appropriately.

Although entrapping elasticity, a physical adsorption process, etc. are mentioned, since the physical adsorption process using a porous carrier is easy to produce, it is preferred. If the microorganism (for example, what is called mold) which has a gestalt of the microorganisms (for example, bacteria, yeast, etc.) which have cohesiveness especially, the shape of flocks, or film state is used, an immobilized microorganism will be obtained only by cultivating with a porous carrier. When using a microorganism as a direct enzyme agent, when an enzyme and a substrate contact efficiently and raise reaction velocity, it will be in a desirable state by processing acetone, alcohol, etc.

[0036]Although there is no restriction especially as ester, as typical ester, fatty acid

ester, especially fats and oils are mentioned. As fats and oils, glyceride and the fats and oils which contain many triglyceride especially are preferred, and vegetable oil and fat, animal fat and oil, fish oil, the fats and oils that a microorganism produces, these mixed fats and oils, or these waste oil is used. As vegetable oil and fat, soybean oil, oleum rapae, palm oil, olive oil, etc. are mentioned. As animal fat and oil, beef tallow, lard, whale oil, mutton tallow, etc. are mentioned. As fish oil, sardine oil, a tuna oil, a cuttlefish oil, etc. are mentioned. As fats and oils which a microorganism produces, the fats and oils produced by the genus *Mortierella* (*Mortierella*), *Schizochytrium* (*Schizochytrium*), etc. are mentioned.

[0037]Waste oil says the fats and oils used for uses, such as food manufacturing, for example, tempura waste oil etc., for example. Waste oil contains the fats and oils by which the hyperoxidation was hydrogenated or carried out, when put to an elevated temperature, but these can also serve as a raw material of biodiesel fuel.

[0038]As alcohol, polyhydric alcohol, such as branching alcohol, such as linear alcohols, such as methanol, ethanol, propanol, and butanol, isopropanol, isobutanol, and 2-butanol, and glycerol, is mentioned. In biodiesel fuel manufacture, it is preferred to use cheap alcohol, such as methanol and ethanol.

[0039]What is necessary is to generally perform more preferably 5 \*\* – 80 \*\* of ester exchange reactions [ 15 \*\* – 50 \*\* of ] between fats and oils and alcohol at 25 \*\* – 45 \*\*, and for the lipase to be used just to determine these, and if it is heat-resistant lipase, a reaction will advance by relatively high temperature.

[0040]Under the above-mentioned reaction condition, an ester exchange reaction advances under existence with ester, alcohol, esterase, and water. When using fats and oils, fatty acid ester and glycerin arise by making fats and oils and alcohol react under existence with water and lipase. This reaction may advance irreversibly. It dissociates from glycerin or unreacted glyceride, and the obtained fatty acid ester is recovered by separating operation, such as settlement, centrifugal separation, membrane separation, molecular distillation, and superfractionation.

[0041]

[Example]Hereafter, although working example is given and this invention is explained, this invention is not limited to this working example.

[0042](Working example 1) The enzyme 1.0g of marketing given in Table 1 was dissolved in 5 ml of distilled water, and enzyme liquid was prepared.

[0043]0.5 ml of enzyme liquid, 4.83 g of olive oil, and 170 mg of methanol were mixed to 20 ml of glassware with a cap, and shake and stirring were performed at 25 \*\*. Under [ a fixed quantity / methyl ester / of the fatty acid produced with gas chromatography

16 hours after the reaction start ].

[0044]The conditions of gas chromatography were as follows.

column; -- DB-5 (J & W Scientific, 10 m x 25 mm)

Initial column temperature; 150 \*\* (0.5 minute)

heating-rate; -- a part for 10 \*\*/-- terminal temperature; -- 300 \*\* (3 minutes)

injector temperature; -- 245 \*\* detector temperature; -- 320 \*\* carrier gas; --

helium (a part for 2.5-cm/)

Spirit ratio; 1/100 [0045]A result is shown in Table 1. The conversion in Table 1 is expressed with the mole ratio of the produced fatty acid methyl ester and the added fats and oils (olive oil).

From the mole ratio of the fats and oils and methanol which were added, 33.3% of conversion means theoretically that the ester exchange reaction advanced 100%.

It is because having carried out 1 / 3 molar-quantity addition of the methanol to fatty acid took into consideration inhibition of the lipase activity by methanol.

[0046]

[Table 1]

酵素名	起源	製造メーカー	反応率(%)
リパ-ゼ QL	<i>Alcaligenes sp.</i>	名糖産業	31.7
リパ-ゼ A	<i>Aspergillus niger</i>	天野製薬	12.9
リパ-ゼ L	<i>Candida lipolytica</i>	天野製薬	31.1
リパ-ゼ OF	<i>Candida rugosa</i>	名糖産業	35.1
リパ-ゼ M	<i>Mucor javanicus</i>	天野製薬	26.3
リパ-ゼ G	<i>Penicillium camembertii</i>	天野製薬	3.5
リパ-ゼ R	<i>Penicillium roqueforti</i>	天野製薬	17.5
リパ-ゼ PN	<i>Phycomyces nitens</i>	和光純薬	13.7
リパ-ゼ P	<i>Pseudomonas alcaligenes</i>	ナガセ生化学工業	32.7
リパ-ゼ AH	<i>Pseudomonas cepacia</i>	天野製薬	30.8
リパ-ゼ AK	<i>Pseudomonas fluorescens</i>	天野製薬	13.3
リパ-ゼ P 原末	<i>Pseudomonas sp</i>	ナガセ生化学工業	15.6
リパ-ゼ PS	<i>Pseudomonas cepacia</i>	天野製薬	27.6
リリパ-ゼ A-10FG	<i>Phizopus japonicus</i>	ナガセ生化学工業	39.4
ニューラ-ゼ F	<i>Rhizopus niveus</i>	天野製薬	19.6
リパ-ゼ F	<i>Rhizopus oryzae</i>	天野製薬	37.1
SM 酵素	<i>Serratia marcescens</i>	ナガセ生化学工業	32.5

[0047]The result of Table 1 shows that the ester exchange reaction advanced, even if water is contained about 9% of the weight. Especially RIRIPAZE A-10FG, the lipase F, the lipase OF, the lipase P, the lipase QL, the lipase L, the lipase M, the lipase AH, and the lipase PS and SM. The ester exchange reaction advanced with high yield, without decomposing the fatty acid methyl ester produced in spite of existence of water.

[0048](Working example 2) The ester exchange reaction in a hydrous system was performed using the filamentous bacterium and yeast which are shown in Table 2.

First, the filamentous bacterium cultivated 37 \*\* of yeast for 37 \*\* and two days 1.0% of yeast extract, and peptone 3.0% on the 1st by pH 6.0 culture medium containing 1.0% of olive oil. 25 ml of each culture medium was freeze-dried, the enzyme agent obtained by preparing an enzyme agent was dissolved in the distilled water of 500microl, and the enzyme solution was prepared.

[0049]The enzyme solution of 500microl, 4.83 g of olive oil, and 170 mg of methanol were added to 20 ml of glassware with a cap, and shake and stirring were performed at 25 \*\*. The thin layer chromatography (TLC) which uses the silica gel 60 (made by Merck Co.) 16 hours after a reaction start considered generation of fatty acid methyl ester. TLC is performed by developing solution:hexane / ethyl acetate / acetic acid =90:10:1, sprayed and heated methanol/sulfuric acid =50/50, and made it color after deployment.

[0050]A result is shown in Table 2. As for +, the conversion as used in Table 2 means that conversion is [ ++ / conversion / +++ / ++++ ] not less than 25% in conversion 18 to 25% 10 to 18% 2 to 10%.

[0051]

[Table 2]

No.	菌株名			反応率
1	<i>Aspergillus</i>	<i>flavus</i>	JCM2061	+++
2	<i>Aspergillus</i>	<i>nigar</i>	JCM5546	+
3	<i>Aspergillus</i>	<i>nigar</i>	JCM1864	+
4	<i>Galactomyces</i>	<i>geotrichum</i>	JCM1945	+++
5	<i>Geotrichum</i>	<i>candidum</i>	IFO4597	+++
6	<i>Mucor</i>	<i>javanicus</i>	IFO4569	+
7	<i>Mucor</i>	<i>javanicus</i>	IFO4572	+++
8	<i>Phycomyces</i>	<i>nitens</i>	IFO5694	+
9	<i>Phycomyces</i>	<i>nitens</i>	IFO9421	++
10	<i>Rhizopus</i>	<i>chinensis</i>	JCM5555	+++
11	<i>Rhizopus</i>	<i>delemar</i>	JCM5564	+++
12	<i>Rhizopus</i>	<i>microsporus</i>	IFO8631	++
13	<i>Rhizopus</i>	<i>oryzae</i>	AHU8537	++++
14	<i>Rhizopus</i>	<i>oryzae</i>	IFO4697	+++
15	<i>Rhizopus</i>	<i>oryzae</i>	IFO6155	++
16	<i>Rhizopus</i>	<i>oryzae</i>	IFO4758	++++
17	<i>Candida</i>	<i>colliculosa</i>	JCM2199	++
18	<i>Candida</i>	<i>parapsilosis</i>	JCM1618	+++

[0052]It was shown that the ester exchange reaction advanced also under the existence of about 9 % of the weight of water.

[0053](Working example 3) The lipase F(product made from Amano Pharmaceuticals) 2.9g was dissolved in 10 ml of distilled water. The 3 ml was used as an enzyme solution, on the other hand, it mixed with 0.8 g of cerite 545, and the 3 ml was fixed. The ester



exchange reaction was performed using the soybean oil 28.95g, the methanol 1.05g, 3 ml of enzyme solutions, or the above-mentioned immobilized enzyme. A result is shown in drawing 1.

[0054]In drawing 1, \*\* expresses the lipase F which is not fixed and — expresses the fixed lipase F. The ester exchange reaction advanced irreversibly and fatty acid methyl ester was generating almost quantitatively the enzyme which is not fixed after the reaction for one day, and the fixed enzyme. further — the methanol 1.05g — having added (1st addition) — time — a reaction — almost — quantitative — having gone on — since — further — methanol — 1.05g — having added (2nd addition). The reaction advanced further by this addition and fatty acid methyl ester was obtained almost quantitatively.

[0055]This result shows that the knowledge of this invention is knowledge which was not considered at all conventionally that an ester exchange reaction (ester synthetic reaction) progresses about 100% under existence of water.

[0056](Working example 4) The influence of the moisture concentration in the system of reaction was considered using the lipase F. Immobilized enzyme was prepared by the same method as working example 3, the soybean oil 28.95g and the methanol 1.05g were mixed, and the ester exchange reaction was performed, the inside of reaction mixture — distilled water — respectively — 3.0 ml, 2.4 ml, 1.8 ml, 1.2 ml, and 0.3 ml — and 0.1 ml added. The conversion rate of the 24th hour is shown in Table 3 after a reaction start.

[0057]

[Table 3]

蒸留水添加量	変換率(%)
3.0ml	33
2.4ml	34
1.8ml	30
1.2ml	20
0.3ml	17
0.1ml	4

[0058]When moisture exceeded about 6 % of the weight, the high conversion rate was attained, but when less than 0.3 % of the weight, the conversion rate was not so higher than Table 3.

[0059](Working example 5) 70 g/l of poly peptone,  $\text{NaNO}_3$  1 g/l, 100 porous carriers of the polyurethane foam (Bridgestone Corp. make; formal HR-50) of 100 ml of culture media (pH 5.6) containing  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g/l, and 20 g/l of olive oils and 6-mm rectangular shape are put into a 500-ml flask, Inoculation of Rhizopus ORIZE

(*Rhizopus oryzae*) IFO4697 was carried out, shaking culture was carried out for 90 hours, 37 °C of biomasses were fixed in the porous carrier, and the immobilized cell was obtained. After it collected these immobilized cells and acetone washed twice, vacuum drying was performed and the fixed dried cell which has lipase activity was prepared.

[0060]The soybean oil 9.65g, the methanol 0.35g, and 50 fixed dried cells that prepared [ above-mentioned ] were mixed to 20 ml of glassware with a cap. 1 ml of distilled water was added to this mixed liquor, and shake and stirring were made to react by carrying out at 30 °C. 0.35g consecutive addition of the methanol is carried out the 1st day and two days after a reaction start, respectively, and it was made for soybean oil and methanol to serve as equimolar mostly eventually. A result is shown in Table 4.

[0061]

[Table 4]

反応時間	変換率(%)
1日	33
2日	62
3日	80
4日	97

[0062]This result shows that it can react efficiently, even if it uses the microbial cell containing lipase as a direct enzyme agent.

[0063]

[Effect of the Invention]Although conventionally reacted by excluding water as much as possible in an ester exchange reaction, it was discovered that an ester exchange reaction is performed and the system which fully contained water can also be performed almost quantitatively. The acyl rearrangement reaction of esterase, especially lipase advances about 100% under existence of water.

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[Translation done.]